Universidade do Algarve

Faculdade de Engenharia e Recursos Naturais

Penicillin G Acylase Encapsulation Studies in Polyvinyl Alcohol Based Matrices

Mestrado em Biotecnologia

Filipa Cristiana Duarte Fangueiro Maduro

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Acknowledgments

My desire to study biotechnology led me to *Universidade do Algarve* where I enrolled in a Master course. This *Mestrado em Biotecnologia* was lectured in a post working schedule and, during one year, every Thursday afternoon I drove to Algarve in order to occupy my weekend studying. It could have been tiring if it hadn´t been for my colleges and teachers; I thank them for making the school year of 2006/2007 educational as well as friendly and funny. For the research work I hoped to stay closer to my workplace, in order to maximize the time spent in the laboratory, so I applied for Institute for Biotechnology and Bioengineering (IBB) as studying in an institution like Instituto Superior Técnico was in many ways appealing. Professor Doutor Joaquim Cabral met me and accepted me to work there for the following year; I would like to thank him for giving me this opportunity. My Supervisor in IBB was Professor Doutor Luís Fonseca which I thank, for taking me as a student but especially for being such a pleasant and human person, he made me feel "at home" and I believe that this has positive repercussions in ones work. To Professora Doutora Deborah Power I thank her help every time I needed, which was always effective. Professor Doutor Pedro Fernandes was always available for my questions and his suggestions where very helpful in guiding my work - Thank You. Susana Bernardino is working in the same field as my research topic and I thank her for her experimental suggestions, her help with the materials and procedures and also her help in the conclusion of this thesis. Daniel Guedelha is a graduate student that was with me for one month and I thank him for making my first experience, in guiding a student, a positive one, which will certainly make me a better "teacher" in the future. Thanks to all my colleagues in IBB for the friendly environment. To Pedro Pinheiro I would like to thank all his Love and moral

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Key-words: penicillin G acylase; β-lacatm antibiotics; cefalexin; immobilization; polyvinyl alcohol; Lentikat® liquid.

Penicillin G Acylase Encapsulation Studies in Polyvinyl Alcohol Based Matrices

Supervisors: Doutor Luís Pina da Fonseca

Doutora Deborah Mary Power

Juri: Doutor José Manuel Peixoto Teixeira Leitão

Doutora Deborah Mary Power

Doutor Luís Joaquim Pina da Fonseca

Doutor Pedro Carlos de Barros Fernandes

Abstract Penicillin G acylase (EC 3.5.1.11) (PGA) hydrolyses penicillin G (PG) producing 6-aminopenicillanic acid (6-APA), an important building block in the synthesis of semi-synthetic antibiotics such as ampicillin and amoxicillin. The synthesis of these antibiotics by PGA is already successfully used in lab and pilot scales and more recently in the synthesis of cefalexin (CEX) at industrial scale. This work characterizes the stability and activity of PGA when immobilized in a polyvinyl alcohol based matrix (Lentikat® liquid), PVA/LL by a new and simple entrapment strategy. The biocatalyst retains from 22 to 66% of its original activity, depending on enzyme load. Hydrolysis and synthesis reactions were tested and the immobilized enzyme can be reused for, at least, 10 consecutive batches without decay of initial activity. The preferential pH and temperature for the hydrolysis of PG are 8 and 37ºC, respectively, it is stable at 4ºC and its half-life, at 25ºC and pH 8 is roughly 8 days. For the synthesis of CEX there was no decay of initial activity for at least 50 hours, at 14ºC and pH 7.2, which indicates a high operational stability. Substrate inhibition by 7-aminodesacetoxicefalosporanic acid (7-ADCA) was observed both for the free and the immobilized enzyme. These forms of enzyme presented synthesis of CEX and hydrolysis of PGM ratio (S/H) of 4.2 and 2.5, respectively. The biocatalyst (PGA_PVA beads) is suitable for the production of cefalexin in industry since it showed a high operational stability, without enzyme leakage during the reaction and between washing steps.

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Introduction

β-lactam antibiotics

Antibiotics are substances derived from living organisms capable of inhibiting vital processes for other organisms. In 1928 Alexander Fleming discovered the ability of a microorganism, *Penicillium notatum*, to prevent the growth of *Staphylococcus* (Fleming 1929), ten years passed before the inhibiting factor was successfully isolated by Florey and Chain (1939). Penicillin G (PG) (Figure 1) was isolated, characterized and rapidly used for medical purposes (Bentley 2005).

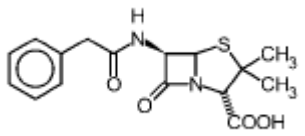

Figure 1 Chemical representation of Penicillin G (Wegman *et al.* 2001).

In 1945 the Nobel Prize in Physiology or Medicine was attributed to Alexander Fleming, Ernst Chain and Howard Florey "for the discovery of penicillin and its curative effect in various infectious diseases" (Raju 1999). The development of the industrial production of Penicillin occurred during the Second World War and its demand was very high. This situation led to a widespread use, which saved many lives of wounded soldiers and later of the general population but this type of use also allowed the appearance of bacteria, that weren't resistant to penicillin, to gain resistance and PG

started to become ineffective towards infections. Bacteria started producing or increasing their production of enzymes that are capable of altering the antibiotic structure and inactivating its biological role.

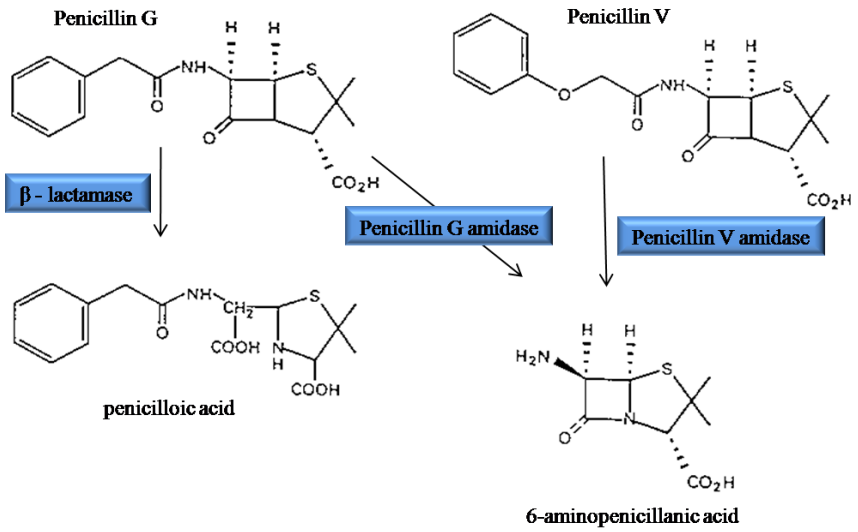

Figure 2 Chemical representation of the reactions that render penicillin a biologically inactive molecule and enzymes that catalyze each reaction.

After its isolation penicillin and other antibiotics were thoroughly studied and by the 1960s Beecham Laboratories had found a way to extract the β-lactam nucleus from penicillin, producing 6-aminopenicillanic acid (6-APA), which derives from penicillin, by the chemical reaction depicted in Figure 3. By using the β-lactam nucleus and altering the acyl donor the function is maintained but bacteria no longer recognize the antibiotic, thus increasing effectiveness. These antibiotics are called semi-synthetic since they are no longer produced by microorganisms but are a chemical modification

of naturally occurring molecules. It is also possible to completely synthesize antibiotics. β-lactam antibiotics are very important in the pharmaceutical industry, with annual sales of ~15bilion, they correspond to 65% of the total antibiotics market (Chandel *et al.* 2008).

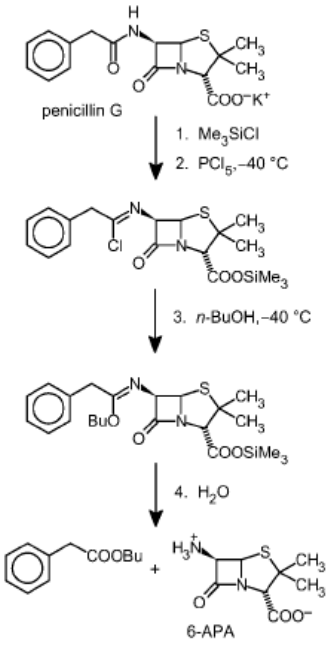

Figure 3 Chemical deacylation of PG into 6-APA (Wegman *et al.* 2001).

Penicillins and Cephalosporins come from fungi, are derived from amino acids and their primary action is to inhibit the biosynthesis of the cell wall of Gram-positive bacteria (penicillins, aminopenicillins, cephalosporins) and Gram-negative bacteria (cephalosporins), impeding peptidoglycan cross linkage by inhibiting the enzyme transpeptidase. There is also an indirect activation effect in hydrolases and autolysins, both these actions contribute to the cell wall destruction, causing the cell to burst when subject to osmotic changes.

Depending on β-lactam nucleus and the acyl derivative linked to it, β-lactam antibiotics can be divided in groups (Table 1).

Table 1 Example of some β-lactam antibiotics and their structural classification.

β-lactam nucleus	Group	Acyl side chain	Name	Semi-synthetic (Ss) / Natural ocouring (No)
6-APA	Penicillins	Ethylphenyl	Penicilin G	No
	Aminopenicillins	D-Phenylglycyl	Ampicillin	Ss
		D-*p*-hydroxy-phenylglycyl	Amoxicillin	Ss
7-ADCA	Cephalosporins	D-Phenylglycyl	Cephalexin	Ss
		D-*p*-hydroxy-phenylglycyl	Cefadroxil	Ss

The most important chemical method for the production of 6-APA was called the "Delft Cleavage" and was a chemical process (Figure 3) used since the 1960's. Very low temperatures are required for this process, spending much energy and hazardous chemicals are used (Wegman *et al.* 2001). At this time Penicillin G acylase (PGA) was known, as well as its capacity to hydrolyze PG, however biotechnology was still in its first steps and the process wasn't economically viable, since the biocatalyst was discarded after use. The usual requirement for a biocatalyst is to achieve product concentrations comparable to chemical processes, given that, in nature, enzymes work at milimolar levels of substrate, it will always be the case that enzymes will be operating away from their natural conditions (Pollard *et al.* 2007). Process development, protein engineering solutions and immobilization techniques evolved and the "Delft Cleavage" was abandoned and completely substituted by the enzymatic pathway

(Figure 4) which is operationally, economically and environmentally more efficient (Kallenberg *et al.* 2005).

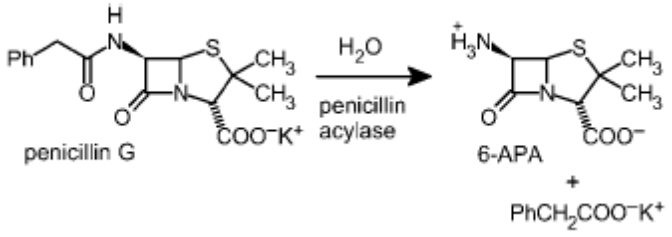

Figure 4 Enzymatic deacylation of PG into 6-APA (Wegman *et al.* 2001).

Nowadays from an industrial point of view, because of bacterial resistance to naturally occurring antibiotics, the penicillin G hydrolysis is very important since its products are the basis for the production of semi-synthetic antibiotics.

Penicillin G acylase

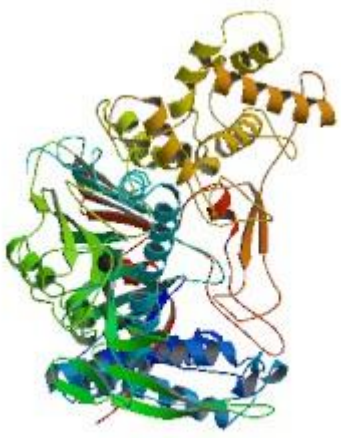

Figure 5 Penicillin G acylase 3D structure by X-Ray diffraction (Duggleby *et al.* 1995).

Penicillin G acylase (EC 3.5.1.11) (PGA) – can be found in a variety of microorganisms, bacteria, actinomycetes, yeasts and fungi. Researchers privileged the isolation of mutants with a high constitutive production of this enzyme, its cloning and its expression in

different hosts to achieve large scale enzyme production (Arroyo *et al.* 2003). The enzyme most studied of the group is Penicillin G acylase cloned in *Escherichia coli* (Janssen *et al.* 2001) and this is the one used for this work.

PGA is responsible for the metabolism of phenylacetic acid (PAA) derivatives that can be used as a carbon source for the organism (Kallenberg *et al.* 2005) and for the defense against antibiotic producing organisms, which gave PGA producing species a clear advantage, it is also responsible for the acquired resistance of some bacteria to penicillin.

Penicillin G acylase from *Escherichia coli* is an oligomeric intracellular enzyme located in the periplasmic space and consists of two different subunits, α and β, with molecular weights of 20.5KDa and 69 KDa, respectively, (Azevedo *et al.* 1999a). The enzyme has a compact structure (pyramidal in cross section) in which the two chains are strongly associated by non-covalent interactions (Brannigan *et al.* 1995).

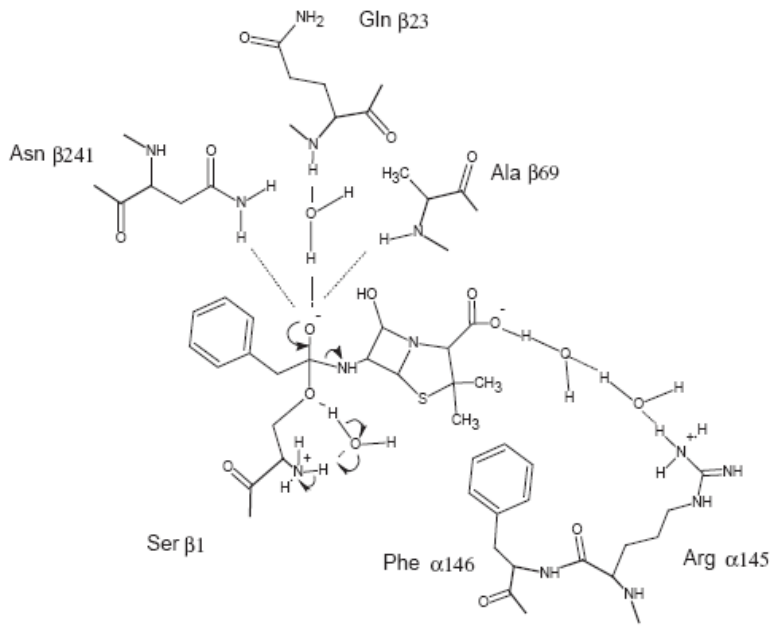

Figure 6 Scheme of the intermediary step of the hydrolysis of PG (Alkema *et al.* 2000).

It has a structural calcium ion and is formed by the processing of a single polypeptide precursor. It's an N-terminal nucleophile hydrolase with a serine residue in its active site (Ser β1) (Figure 6) that promotes a nucleophilic attack to the carbonyl carbon of amide or ester bonds (Giordano *et al.* 2006). Like other N-terminal hydrolases, this enzyme owns a four-layered catalytically active site with a αββα-core structure formed by two antiparallel β-sheets packed against each other, covered by a layer of α-helices on one side (Arroyo *et al.* 2003). X-ray studies indicate that the active site has a roughly conical groove which accommodates the phenylacetyl moiety of the ligands, the hydrophobic part and the more polar aminic part of the residues locate in the open part of the groove, surrounded by several polar amino acid residues (Guncheva *et al.* 2004). The catalytic site is located in the β-subunit and the binding site, that confers specificity for the different substrates, is located on the α-subunit. The acyl binding subsite is highly specific for phenylacetic acid (PAA); only small groups, such as hydroxyl or amino groups, are allowed at the 2-position and the enantiomer specificity is low. The penicillin-recognizing subsite, in contrast, is highly specific for L-amino acids but also discriminates other chiral primary amines (Azevedo *et al.* 1999a).

PGA hydrolyses penicillins and cephalosporins to produce β-lactam antibiotic intermediates like 6-APA and 7-aminodesacetoxycephalosporanic acid (7-ADCA), respectively, with PAA as a common by-product (Chandel *et al.* 2008). The hydrolysis reaction is thermodynamically favorable but, like any other catalyst the inverse reaction - synthesis - is also possible (Figure 7) by the condensation of an acyl donor to a β-lactam nucleus. Controlling the reaction conditions and the substrates used, since PGA is not a very specific enzyme due to its large accessible, conical shaped, active site, it can act preferentially as a synthetase, a hydrolase, an esterase or an amidase. Changing

the acyl donor one obtains different penicillins, or cephalosporins if 6-APA is replaced by 7-ADCA. It can also be used in other potentially valuable reactions such as the protection of amino and hydroxyl groups in peptide synthesis and in the resolution of racemic mixtures, it possesses extremely high stereospecificity in hydrolysis of N-acylated amino compounds (Grinberg *et al.* 2008).

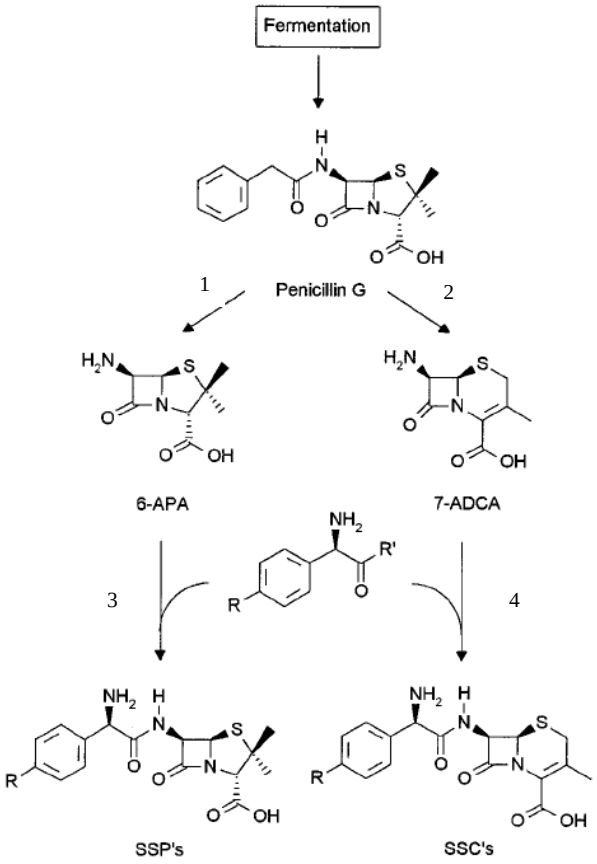

Figure 7 Scheme representing four of the possible reactions catalysed by PGA (Bruggink *et al.* 1998). Were SSP's – semi-synthetic penicillins and SSC's – semi-synthetic cephalosporins.

To produce β-lactam antibiotics thermodynamically or kinetically controlled enzymatic synthesis can be used *via* reversal of the hydrolytic reaction or *via* transacylation,

respectively (Wegman *et al.* 2001). For the thermodynamically controlled synthesis, which proceeds towards equilibrium, to improve the direct synthesis, acids are used to acylate the β-lactam nucleus at low pH values (undissociated acid) and water-organic solvent mixtures are used (Arroyo *et al.* 2003). The organic solvents minimize the activity of water thus minimizing hydrolysis. Recent studies, however, proved that equilibrium is overwhelmingly on the side of hydrolysis due to the low chemical energy of the zwitterionic phenylglycine. For the kinetically controlled synthesis esters or amides are used to lower the activation energy (E_a) of the antibiotic synthesis, the equilibrium overshoots thus favoring the syntethase activity over the hydrolase activity in the beginning of the reaction (Figure 8). The yield of this type of synthesis depends on the rate of synthesis over the rate of hydrolysis of the activated acyl donor and the hydrolysis of the synthesized antibiotic (S/H); the reaction should be stopped when the synthesis rate becomes equal to the hydrolysis rate of the antibiotic and the product, when the concentration of SSA passes through a maximum, otherwise kinetic control is lost and thermodynamic control takes over. S/H ratio generally declines as the reaction proceeds, due to the transformation of the starting compounds, accumulation of product and change in ionic strength (Wegman *et al.* 2001) so, instead of toping the reaction at maximum conversion it can be stopped at lower conversion values and recycle the starting compounds and in this way working at maximum (S/H). The starting compounds have to be stable and even then the downstream process becomes quite complex. These reactions are performed in water at physiological pH and low temperatures (to minimize the thermodynamic pathway).

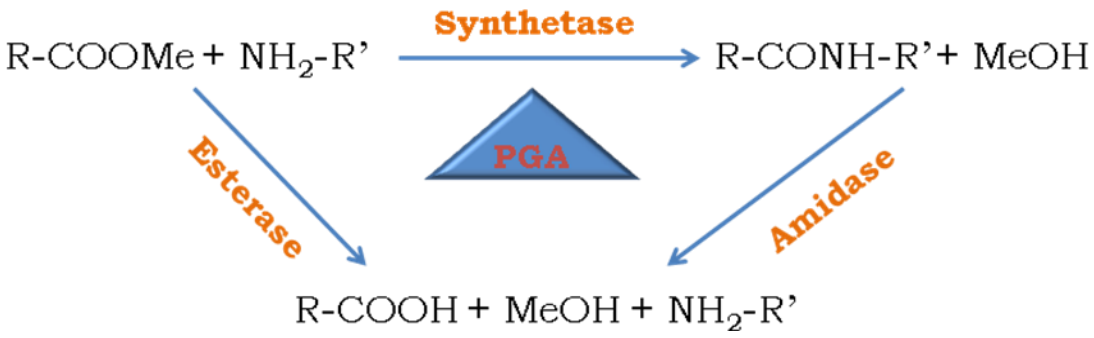

Figure 8 Scheme of the possible functions of PGA when using a kinetically controlled reaction. Addapted from (Fernández-Lafuente *et al.* 1998).

As the production of 6-APA and 7-ADCA enables the production of semi synthetic antibiotics which remain the most widely used group of antibiotics (De Vroom 1999), for they correspond to 65% of the worldwide production of antibiotics, exceeding 45000 tons in 2000 (Giordano *et al.* 2006), PGA is an economically important enzyme. Its demand is being met by a fermentation process that uses genetically manipulated *Escherichia coli* and *Bacillus megaterium* microorganisms (Chandel *et al.* 2008). Due to the rising interest in sustainable development and environmentally friendly practices, microbial enzyme transformation processes are generally preferred over the conventional chemical conversion process and an annual worldwide production of 9000 tons of 6-APA are produced enzymatically from Penicillin G and V.

Immobilization of enzymes

Recently the world gained awareness to the importance of sustainable development and imposes its practice by the industry. In the search for environmentally friendly

practices, microbial enzyme transformation processes are generally preferred over the conventional chemical conversion process, replacing classical organic methods by cleaner catalytic alternatives that minimize the generation of waste and avoid the use of toxic and hazardous chemicals. Biocatalytic procedures are usually performed in mild reaction conditions, at neutral pH and temperature close to ambient, they use a environmentally friendly solvent, water and a biodegradable catalyst (Wegman *et al.* 2001). In addition to being environment friendly, enzymes also possess high activity, chemo-, regio- and stereo- selectivity which makes them the best choice to lower the number of steps in a chemical process by eliminating functional group protection / activation permitting to greatly simplify the design of the reactor and the control of the reaction: the simple filtering of the enzyme stops the reaction (Sheldon 2007).

Some factors that adversely affect the usage of enzymes are their high cost, relative instability under industrial conditions, limited specificity and competition with established chemical processes carried out in equipment with fully depreciated capital cost (Zaks *et al.* 1997). Enzymes are expensive catalysts for their purification is laborious - the purer the enzyme preparation, the more expensive it becomes. To use biocatalysts in an economical viable way is important that it is stabilized and that it may be reused and recovered. Immobilization is the preferred way to use enzymes as industrial biocatalysts and this is the simplest solution to their solubility problem (Mateo *et al.* 2007).

To achieve a robust immobilized enzyme the method has to be suitable (carriers, conditions, and enzymes) to design a biocatalyst that can meet the catalytic parameters (expressed as productivity, space–time yield, stability and selectivity) and the

noncatalytic ones (e.g. separation, control, down-streaming process) of a given application (Cao 2005).

The idea to immobilize enzymes came to Nelson and Griffin in 1916 when they observed that artificial carrier-bound invertase on $Al(OH)_3$ and charcoal was still catalytically active (Nelson *et al.* 1916). A long time passed before their work was recognized as an important discovery in chemistry. Nowadays immobilization is a widely developed field and the most common immobilization methods can be divided in four major groups: Adsorption-based, Covalent Immobilization, Entrapment and Encapsulation (Figure 9).

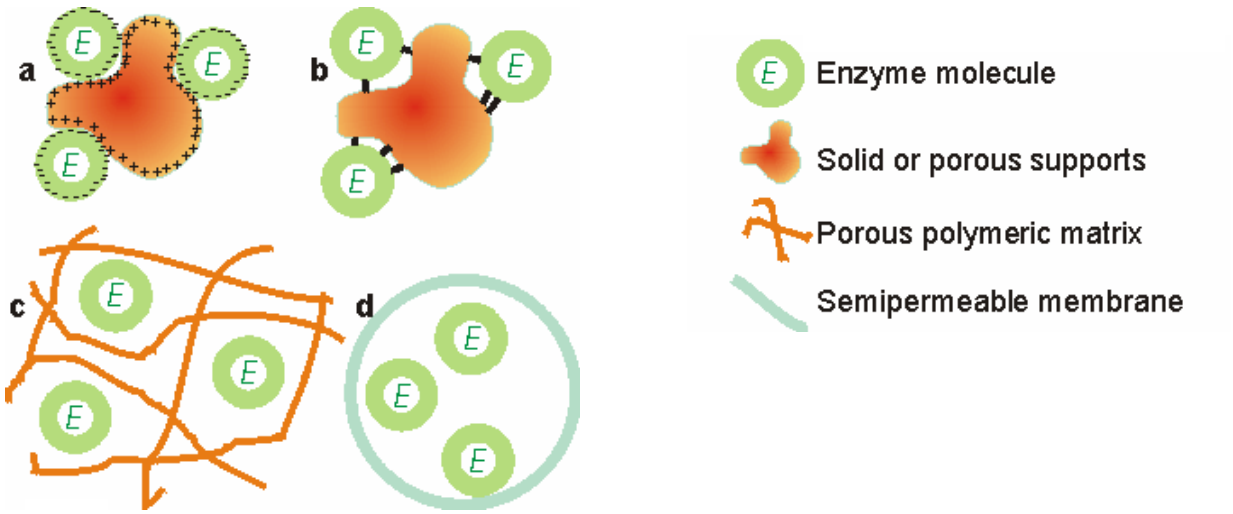

Figure 9 Representation of four different immobilization techniques: (a) Adsorption-based immobilization; (b) Covalent immobilization; (c) Entrapment and (d) Encapsulation.

- The first method is based on the physical adsorption of an enzyme on the surface of water-insoluble carriers. Hence, the method causes little or no conformational change of the enzyme or destruction of its active site. A major advantage of

adsorption as a general method of immobilizing enzymes is that usually no reagents and only a minimum of activation steps are required. The binding is mainly by hydrogen bonds, multiple salt linkages, and Van der Waal's forces. Regarding this idea, the method bears the greatest similarity to the situation found in natural biological membranes and has been used to model such systems, due to the weak bonds involved, desorption of the protein is often observed. Another disadvantage is non-specific, further adsorption of other proteins or other substances as the immobilized enzyme is used.

Ionic binding is a type of adsorption-based immobilization that relies on the ionic binding of the enzyme to water-insoluble carriers containing ion-exchange residues. The main difference between ionic binding and physical adsorption is that the enzyme to carrier linkages are much stronger for ionic binding although weaker than in covalent binding.

- The covalent binding method is based on the binding of enzymes and water-insoluble carriers by covalent bonds. The binding reaction must be performed under conditions that do not cause loss of enzymatic activity and the active site of the enzyme must be unaffected by the reagents used. Covalent binding may alter the conformational structure and active site of the enzyme, resulting in major loss of activity and/or changes of the substrate. However, the binding force between enzyme and carrier is so strong that no leakage occurs. Immobilization of enzymes has been achieved by intermolecular cross-linking of the protein, either to other protein molecules or to functional groups on an insoluble support matrix. Cross-linking an enzyme to itself is both expensive and insufficient, as some of the protein material will inevitably be acting mainly as a support. This will result in

relatively low enzymatic activity. Generally, cross-linking is best used in conjunction with one of the other methods. It is used mostly as a mean of stabilizing adsorbed enzymes and also for preventing leakage from polyacrylamide gels.

- Entrapment methods involve trapping enzymes within the interstitial spaces of a cross-linked water-insoluble polymer. The entrapped enzyme is not supposed to attach to the polymer, its free diffusion is impeded by physical restraints caused by the lattice structure of the gel, thus minimizing loss of activity. Some synthetic polymers such as polyacrylamide, polyvinylalcohol and natural polymers (starch) have been used to immobilize enzymes using this technique. The advantage of using entrapment as an immobilization technique is that this method is fast, cheap, simple and usually involves the use of mild reaction conditions. The usual disadvantage of this method is that, due to the reduced size of enzymes, leakage often happens; it is usually used to immobilize whole cell systems. The porosity of the matrix can be controlled to prevent leakage, yet at the same time it has to allow free movement of substrates and products. Depending on the price of the enzyme and its productivity when immobilized, this method may or may not be economically viable for industrial application of enzymes.
- Encapsulation involves enclosing the enzymes within semi-permeable polymer membranes. It is similar to entrapment in the sense that enzymes are free in solution but restricted in space. They must confine the enzyme whilst allowing free passage for the reaction products and, in most configurations, the substrates. The preparation of enzyme micro capsules requires extremely well-controlled conditions. Many materials have been used to produce microcapsules being the

most popular nylon and cellulose nitrate but also unconventional methods have been developed like the use of Erythrocytes (Bickerstaff 1997). Again problems associated with diffusion occur and the membrane can rupture if products accumulate rapidly in the interior. An advantage of this procedure is that different enzymes can be co-immobilized, forming a multienzymatic system and a chemoenzymatic cascade process.

After the acknowledgment that enzymes can be the answer to the viable application of green chemistry, and, as they need to be immobilized, for that to happen many different techniques where developed. Today the variety of immobilization matrices makes it possible to choose a ready-made immobilization method, considering the enzyme characteristics. Nevertheless there are still many more methods being developed for general use and for a specific type of enzyme and reaction. The first possibility is obviously less desirable, because a ready-made immobilized enzyme is not necessarily the optimum catalyst for the desired processes, as exemplified by the fact that many types of carrier-bound immobilized penicillin G acylase, which are regarded as robust immobilized catalysts for the production of 6-APA, are not necessarily good catalysts for the kinetically controlled synthesis of semi-synthetic β-lactam antibiotics. This is largely ascribed to the fact that changing the process conditions often provokes a change of enzyme performance. It was only in the 70s that such variables as the microenvironment effect of the carrier, the effect of the spacer or arm, different modes of binding (chemistry, position and number), enzyme loading, changes in the conformation of the enzyme, diffusion constraints, orientation of the enzyme, and the protective effect of substrate or inhibitor during immobilization, where studied in order to improve the stabilization and the productivity of the immobilized enzyme. Until the

end of the 1980s, incentives to design robust immobilized enzymes originated from their positive potential aspects, another important incentive was the discovery that many enzymes are catalytically active and stable in organic solvents, thus enabling many reactions which cannot be performed in aqueous media (Cao 2005). The Immobilization of enzymes also brings advantages in the process design. The biocatalyst is retained inside the reactor which allows its reutilization or continuous flow in a packed-bed or fluidized-bed, if its stability is high enough, and diminishes wash-out problems; high concentrations of biocatalyst can be used, which accelerates production, increases productivity and minimizes secondary reactions; the microenvironment can be controlled and it is easily separated from the product; the enzyme molecules are protected from aggregation autolysis and proteolysis, they are also protected from external hydrophobic interfaces such as air bubbles.

Along with the advantages, immobilization characteristics introduce changes and new limitations to the reaction flow, there is loss of activity:

- during the immobilization steps, due to exposure of variations in pH, temperature or others necessary for solidification or to distortions of the enzyme shape when the matrix is solidifying;
- due to the matrix, the lattice arrangement excludes macromolecules, induces local pH variations, stereochemical hindrances in active site access and mass transfer limitations;
- during the bioconversion due to leakage of the enzyme, chemical interactions between the matrix and solutes, pores of different sizes, accumulation of inhibitors, toxic agents or contaminants and solid retention;

- and the method's development is still empirical which demands optimization studies for each case and makes the modulation and process control more difficult (Cabral *et al.* 2003).

If the enzyme is multimeric it may dissociate in its individual subunits and inactivation may occur, since after immobilization there is no free movement the enzyme can't reassemble (Mateo *et al.* 2007).

Isolated enzyme processes are simple to implement because they are amenable to rapid process development and there are few side reactions or other metabolites that require separation from the reaction product. However the price to be paid for these benefits is higher upstream costs and enzyme reuse, which is essential to keep these costs manageable. The aim is to minimize the enzyme purification and operate the process with the crudest form of the catalyst possible (usually a lysate). It is noteworthy that many academic enzyme catalysts studies are carried out using pure enzymes and their results might not always be easily translatable at scale (Pollard *et al.* 2007). Immobilized enzymes are already industrially used in different fields such as medicine, chemistry, food and beverages and waste treatment. An application of immobilized enzymes, that greatly simplified the chemical pathways, was the separation between chiral molecules in a racemic mixture. This was the first industrial application of an immobilized enzyme, aminoacylase immobilized in DEAE-sephadex by Tanabe Seiyaku Company; Japan was used for the resolution of racemic mixtures of chemically synthesized amino acids. The conversion of glucose syrups to high fructose syrups is achieved by glucose isomerase either adsorbed to an insoluble surface or cross-linked, different companies developed and use different immobilization techniques. Thousands of tones of high – fructose syrup are produced annually by this enzyme process, which

is the most widely used of all the immobilized enzyme systems. Another viable industrial application of immobilized enzymes is the use of lactase to hydrolyze lactose from dairy products, since a large percentage of the world population gradually becomes lactose intolerant this technique allows milk consumption for longer periods of time, increasing sales. The first company to apply this method was Centrale del Latte of Milan, Italy, utilizing the Snamprogetti technology.

In the pharmaceutical industry one of the major applications of immobilized enzymes is the production of 6-APA by the deacylation of the side chain of Penicillin G or V, catalyzed by PGA, for the production of semi-synthetic antibiotics. One of the advantages of the enzymathic pathway is that purer product is obtained, thus minimizing the purification costs, along with the previously mentioned advantages. In the 1970s Squibb (USA), Astra (Sweden) and Riga Biochemical Plant (USSR) started using this setup simultaneously. Currently most of the pharmaceutical giants make use of this technology. Similar approaches have also been used for the production of 7-ADCA, an intermediate in the production of semis-ynthetic cephalosporins (D'Souza 1998). Immobilized PGA is also used by Chemferm, a subsidiary of DSM, Netherlands, for the production of cefalexin, which is for now the only biochemically synthesized β-lactam antibiotic (Wegman *et al.* 2001). Eli Lilly, United states, produces a slightly altered cefalexin molecule (Figure 10), which sells by the trade name Locarbef (Zaks *et al.* 1997).

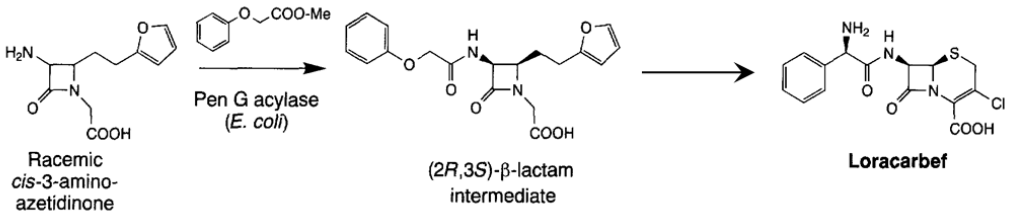

Figure 10 Synthesis of Locarbef. (Zaks *et al.* 1997)

Immobilization of Penicillin G acylase

PGA immobilization was thoroughly studied and a number of different systems have been patented, either isolated enzymes or whole cells are used. Different authors reviewed the state of the art of biotechnological applications of Penicillin acylases (Kallenberg *et al.* 2005), (Arroyo *et al.* 2003), (Wegman *et al.* 2001), (Chandel *et al.* 2008), (Giordano *et al.* 2006) and bellow is shown a summary of the most important systems, based mainly on these reviews.

Physical adsorption of PGA is not very used since the enzyme is very expensive and these applications have the drawback of leakage; nonetheless there is still a couple worth mentioning. A biopolymer of activated cellulose beads followed by covalent attachment of Concavalin A (1% wt) absorbed a PGA-mannan conjugate and retained an activity yield of 74%, however the procedure is complex and the activity of the resulting biocatalyst modest. Celite® is a trade name for diatomite, a silica material that consists of the skeletons of diatoms (unicellular algae that may live in colonies in the form of filaments). Up to 300 U of penicillin G acylase were absorbed per g of Celite R-640. The activity recovery was low, approx. 45 U g^{-1}, which corresponds with an activity yield of 17%. This procedure was one of the first examples of successful use of

penicillin G acylase in an organic solvent; the preparation maintained a useful activity in toluene, provided that a water activity > 0.4 was maintained.

Covalent attachment of PGA to an insoluble support is the most used method to immobilize this enzyme. This method diminishes diffusional restrictions to substrate or product, the rigid enzyme-support linkage provides both kinetic and thermodynamic stabilization of the three dimensional structure of the active catalytic site and often improves the enzyme thermal stability. The general problem with covalent binding is the lack of active sites, low immobilization yield and low final activity. Eupergit® C immobilized PGA by multipoint covalent attachment, which is considered a major contributing factor to the high operational stability of enzymes and 85-90% of immobilization yield was achieved. This is a highly hydrophilic material which can account for the high yield since PGAs surface is also hydrophilic and the enzyme has a low stability when in contact with a hydrophobic medium. Amberlite XAD-7 is a porous poly (methacrylic ester) that physically adsorbs PGA, but this linkage is not stable, so the resine was activated by aminoalkylation followed by derivatization with a glutaraldehyde linker, 194 Ug^{-1} of activity was recovered, which corresponds to a moderate 25%. If the beads are reduced in size the yield increased to 33%, it has been found in early, related work, that penicillin G acylase had penetrated only a 25 μ layer below the surface of the XAD-7 beads in 24h which can account for the low immobilization yield. Aldehyde-agarose, a linear polysaccharide, in which enzymes can be linked by multipoint covalent attachments, has been extensively studied in the group of Guisán. In this support PGA retained 80% of its original activity, which is a very good yield the drawback is that agarose is an expensive polymer (Adriano *et al.* 2005). Penicillin G acylase was covalently attached to activated delaminated pure silica ITQ-6

(a delaminated zeolite) and stabilized by reduction of the imine bonds to secondary amines. The activity of the resulting preparation was 2440 Ug^{-1} that is the highest ever reported for a carrier bound penicillin G acylase which can be ascribed to the highly accessible structure with a high surface area and large pores. A recent approach to immobilization of enzymes is the covalent attachment to stimulus-responsive or 'smart polymers' which undergo dramatic conformational changes in response to small changes in their environment, e.g., temperature, pH and ionic strength (Sheldon 2007). PGA was immobilized by condensation with a copolymer of NIPAM containing active ester groups. The resulting enzyme-polymer conjugate exhibited hydrolytic activity close to that of the free enzyme and was roughly as effective as the free PGA in the synthesis of cephalexin (Sheldon 2007). There are many other methods to covalently immobilize PGA that aren't mentioned by the authors above, like covalent immobilization of PGA on Sepabeads EC-EP carrier, an epoxy activated polymetacrylic carrier with an immobilization yield of 89.4 % at 0.14 $mg_{enzyme}g^{-1}_{support}$ (Žuža *et al.* 2007) or covalent immobilization to Poly(vinyl acetate-co-diviyl benzene) beads (Jianguo *et al.* 2001).

Entrapment of enzymes may be difficult due to enzyme/pore size relation, for it is frequent that enzymes leak from the matrix into the reaction medium. The most common use for this technique is the immobilization of whole cells or the entrapment of covalently immobilized enzymes in hydrophilic polymers so that side reactions or inhibition by organic solvents are minimized. Polyvinyl alcohol (PVA) crosslinked with boric acid was used to immobilize whole cell PGA from *Alcaligenes faecalis* expressed in *Escherichia coli* (Cheng *et al.* 2006). The conversion yield from PG to 6-APA was 75% and there was no activity loss after 15 cycles in batch reaction. Hydrogels, like

PVA, are suitable matrices for the immobilization of enzymes and whole cells; they are cheap, non toxic, hydrophilic, have a low coefficient of friction, and high Water content. PVA based matrices have a considerable chemical and mechanical stability when comparing to naturally occurring polymers (Fernandes *et al.* 2008). A mild and highly efficient method for preparing PVA hydrogels by partial drying at room temperature afforded lens-shaped hydrogels (Lentikats®) exhibiting good mechanical stability, easy separation and stability towards degradation. Lentikats have been successfully used for the entrapment of whole cell biocatalysts or cross-linked enzymes like the encapsulation of cross linked enzyme aggregates (CLEA) of PGA. This method has been used successfully in organic medium to improve the inadequate mechanical properties of CLEA (Wilson *et al.* 2004). There are, however, methods to directly entrap enzymes. In contrast with covalent immobilization on a silica surface, enzymes can also be entrapped within a silica matrix. When PGA was immobilized in silica by entrapment the activity recovery was only 10%. It was reported that penicillin G acylase is inherently more sensitive to diffusion limitation than, for example, a lipase. Gelatin-chitosan entraps PGA it is only 30% efficient in the hydrolysis of PG but later, it was claimed that the pencillin G acylase from *E. coli* performed much better in the synthesis of β-lactam antibiotics when immobilized in gelatin-chitosan than with other supports. This was the basis for the industrial production of Assemblase® which is nowadays used in the enzymatic synthesis of β-lactam antibiotics.

Carrierless enzyme immobilization is potentially advantageous because it saves the costs of the carrier, which surpass those of the enzyme in some cases, and avoids the handling of a large mass of inactive material in the course of the reaction. Such biocatalysts can be packed in a column but are too fragile for use in a stirred batch

reactor. Cross linked enzyme crystals (CLECs) of penicillin G acylase have been commercially available for some time from Altus Biologics but the production of biocatalyst CLECs has been discontinued. Alternative to crystallization, the physical aggregation of protein molecules into super-molecular structures and subsequent precipitation can be induced by the addition of salts, organic solvents or non-ionic polymers to protein solutions, without perturbation of the original three-dimensional structure of the protein. Indeed, precipitation induced by ammonium sulfate, polyethylene glycol, and some organic solvents such as alcohols, is a commonly used method of protein purification. When these solid aggregates are rendered permanently insoluble by chemical cross-linking, cross-linked enzyme aggregates are obtained (CLEAs). Up to 80% of the original activity was recovered after the formation of PGA CLEAs. An active site titration study revealed that loss of active sites and loss of turnover rate contributed equally to the activity loss. (Kallenberg *et al.* 2005) The first examples of CLEAs derived from penicillin G amidase. Penicillin G amidase CLEAs, proved to be effective catalysts for the synthesis of ampicillin. The CLEA exhibited a synthesis/hydrolysis ratio comparable with that of the commercial catalyst, PGA-450 (penicillin G amidase immobilized on polyacrylamide). The PGA CLEAs also maintained their high activity in organic solvents. (Sheldon 2007)

Especially for the thermodynamically controlled synthesis of β-lactam antibiotics the use of organic solvents is important. Thermodynamically controlled synthesis follows the reaction equilibrium and yields are only determined by the thermodynamic constant of the process. To circumvent this problem the reaction conditions can be altered in order to shift the equilibrium in favor of the synthetic pathway. The use of organic solvents increases the pKa of the carboxylic group and decreases water activity, hence

decreasing hydrolysis (Fernández-Lafuente *et al.* 1998). Immobilization is generally necessary for optimum performance of enzymes in non-aqueous media, PGA doesn't only need to be immobilized, but also needs an hydrophilic microenvironment to ensure stability since enzymatic activity is highly dependent on the water content of the system (w_o) (Azevedo *et al.* 1999b). In non-aqueous media enzymes can also be immobilized in natural or synthetic hydrogels or cryogels. Polyvinyl alcohol (PVA) cryogels formed by the freeze-thawing method, for example, have been widely used for immobilization of whole cells.

The chemical and genetic amination of the surface of some enzymes (e.g., glutaryl acylase, penicillin G acylase) have permitted to greatly improve the multipoint covalent attachment of the enzyme, increasing its stability when compared to the native form. In a similar way, chemical and genetic modification of the protein surface has improved the reversible adsorption of penicillin G acylase on both, cationic and anionic exchangers. Penicillin G acylase is an enzyme that suffers a relatively large conformational change during catalysis, due to an acyl-induced exposition of the reactive serine. Its properties have been also modulated by immobilization, both in synthesis of antibiotics and in resolution of racemic mixtures (Mateo *et al.* 2007). Relative rates of hydrolysis and synthesis of b-lactam antibiotics may also be modified by site-directed enzyme mutagenesis. Mutation of the phenylalanine at position 24 in the β subunit to alanine (βF24A) of penicillin G acylase from *E. coli* has yielded a mutant that showed a higher synthesis/hydrolysis ratio, an increased esterase/amidase activity and reduced inhibition by phenylacetic acid in the synthesis of amoxicillin, ampicillin, cefadroxil and cephalexin (Alkema *et al.* 2000).

In spite of the amount of different immobilization techniques, new techniques are sill emerging to successfully immobilize PGA, stabilize it and increase hydrolysis of PG or synthesis of β-lactam antibiotics. With the continuous development of immobilization techniques and protein engineering surely big improvements can be expected in the development of carriers for PGA since it is a versatile enzyme in the production of increased value substances like antibiotics and chiral compounds. The aim of this work is exactly this, to study and characterize a new immobilization method that will contribute to the knowledge of this enzyme and hopefully to the development of new, industrially applicable, immobilization techniques. A modified GeniaLab methodology was used for the encapsulation of a commercial inulinase preparation in PVA capsules. PVA capsules where obtained from LentiKat® liquid upon extrusion into polyethylene glycol (PEG) where gelification occurred instantaneously. The methodology presented is a reminiscence of the classic protocol for immobilization in Calcium alginate gel particles (Fernandes *et al.* 2008). The technique used by Fernandes is a simple and cheap way to immobilize enzymes without the need of prior treatment, this makes it an interesting approach to apply to PGA since in industry simplicity is often as much of a decisive factor for the adoption of new methodologies as product yield.

Materials and Methods

Materials: Penicillin G acylase (EC 3.5.1.11) (PGA) (19 U/mg) and polyethyleneglycol 600 were purchased from Fluka, Penicillin G was obtained from Fersinca Gb (Mexico), 7-aminodesacetoxycephalosporanic acid (7-ADCA) was purchased from DSM, the substrates 6-nitro-3-phenylacetamide benzoic acid (NIPAB), acetaminophen 99% (IS) and (R)-(-)-2-phenylglycine methylester hydrochloride 97% (PGM) were obtained from Sigma and the immobilization matrix, Lentikat® Liquid, was obtained from GeniaLab® GmbH. All other chemicals were either laboratory or analytical grade.

Methods:

1) Penicillin Hydrolysis

1.1. Immobilization Yield

Entrapment technique Six diluted PGA solutions were prepared with 15, 30, 50, 100, 125 and 150µl of original PGA solution for a final volume of 500µl in sodium phosphate buffer 0.1M, pH8. The Lentikat® Liquid (LL) was heated to reach the liquid state and then cooled by placing in a 37ºC bath. When the LL reaches 40ºC, 5ml were used to dissolve each diluted PGA solution under strong magnetic stirring. With a syringe of Injekt – F 1ml Braun and a Neolus (0.9 x 50 mm) Terumo needle the LL/PGA solution was dropped (Figure 11) into 200 ml of PEG 600, the droplets immediately formed capsules and then were left in PEG for two hours at room temperature to solidify under mild magnetic stirring (Fernandes *et al.* 2008). The

PGA_PVA beads were removed by filtration, washed several times with sodium phosphate buffer solution 0.02M, pH 8, weighed and stored at 4ºC in sodium phosphate buffer solution 0.1M, pH 8, just enough to cover the beads. The material that comes in contact with the LL/PGA solution is weighed before and after this process so that mechanical losses of enzyme can be determined.

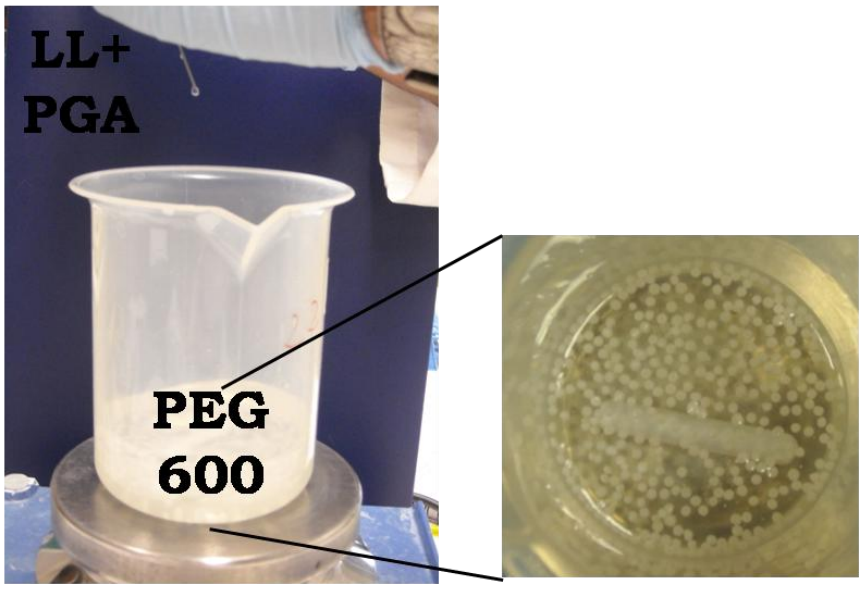

Figure 11 Immobilization technique, dropping the PGA_PVA solution into PEG 600.

Hydrolysis assays To measure the biocatalysts activity the immobilized enzyme beads were suspended in 20ml of sodium phosphate buffer solution 20 mM, pH 8 at 37ºC, in a temperature controlled, magnetically stirred reactor. When the suspension reached 37ºC, 5 ml of concentrated penicillin G solution, in the same buffer at the same temperature, was added so that its final concentration in the reaction media was 4% (w/v). Penicillin hydrolysis was followed during 10 minutes (Figure 13) by the pH STAT method

(Figure 12) (Fonseca *et al.* 1993), using a standard of 0.1M NaOH to automatically neutralize the phenylacetic acid (PAA), using a Metrohm 702 SM Titrino, generated for biocatalysts obtained from 150, 125, 100 and 50µl of enzyme load and a standard of 0.05M NaOH for 30µl and 15µl of enzyme load into LL solution. The beads were harvested and washed in buffer for 10 min, the resulting diluted reaction media supernatant was concentrated by ultrafiltration, using a Cole-Parmer Instrument CO Stirred cell 43 mm (UHP) 70986 and a Biomax – 5, 44.5 mm, 5 KDa polysulfone membrane from Millipore Corporation, until the final volume reached ~5ml to control enzyme leakage at the end of enzyme immobilization process (see supernatant activity). Each assay was repeated three times and in the end of the assays the beads were dried at 80ºC for 24h and weighed, the dry weight was used to standardize the results.

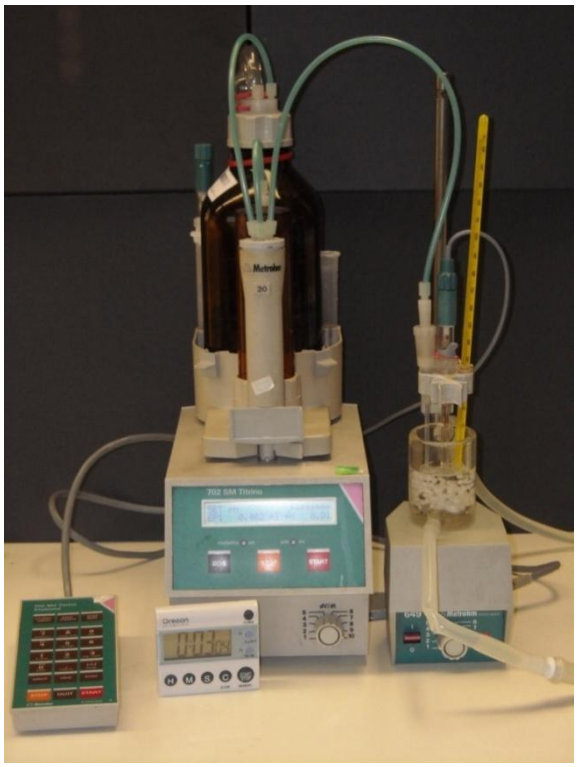

Figure 12 Apparatus to measure penicillin G hydrolysis by the pH STAT method.

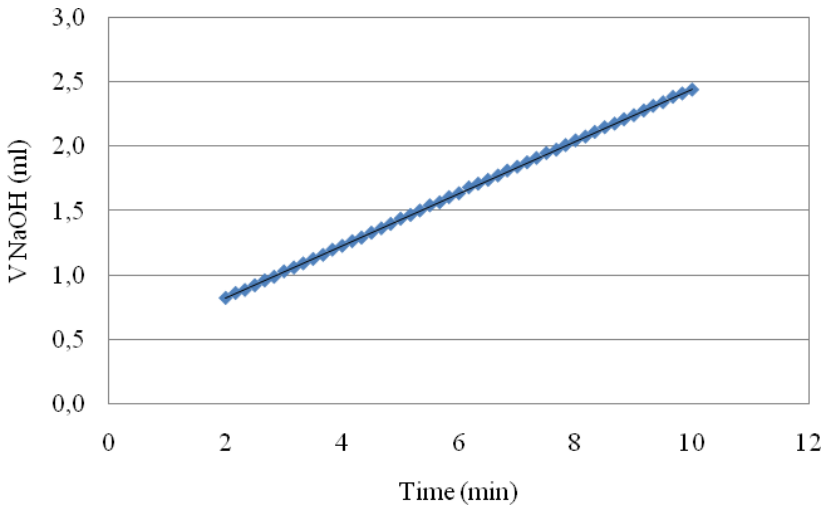

Figure 13 Volume of NaOH spent versus time, result of a typical PGA activity assay by pH STAT.

The supernatant activity was determined in a Hellma, 119.004 – KS, 10 mm cuvette with magnetic agitation, by following the absorbance at 37ºC, 405 nm in a Hitachi U-2000 spectrophotometer. Absorbance is proportional to 3-amino-6-nitrobenzoic acid (NABA) concentration, the product resulting from the hydrolysis of 6-nitro-3-phenylacetamide benzoic acid (NIPAB) by PGA. 50µl of NIPAB 6mM and 50 µl of the enzyme solution to be analyzed was added, respectively into 900µl of sodium phosphate buffer solution 50mM, pH 7 (Azevedo *et al.* 1999b). The activity of PGA solutions, using different degrees of dilution, was measured and used to assess the amount of active free enzyme in the supernatant by hydrolysis of NIPAB (Figure 14).

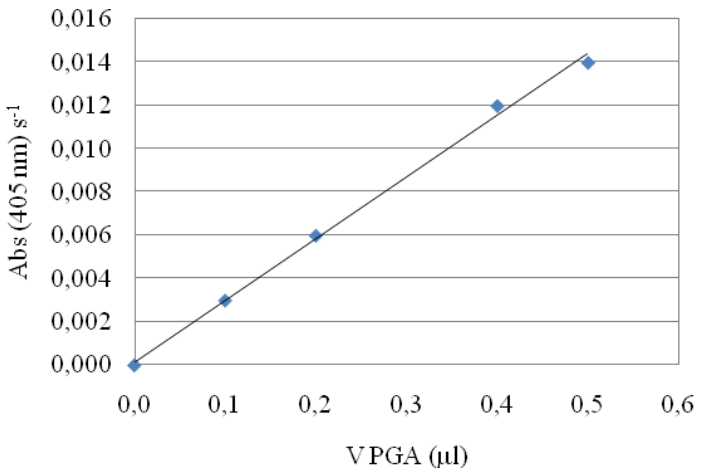

Figure 14 Rate of NIPAB hydrolysis reaction by PGA and according absorbance rate variation at 405 nm for different volumes of supernatant.

Total enzyme in the supernatant The total amount of protein in the supernatant was determined by its absorbance at 280 nm using a Hitachi U-2000 spectrophotometer. A calibration curve was built for absorbance at 280 nm using different amounts of diluted PGA in sodium phosphate buffer solution 50mM, pH 8 (Figure 15). The reaction media supernatant solution was analyzed without dilutions.

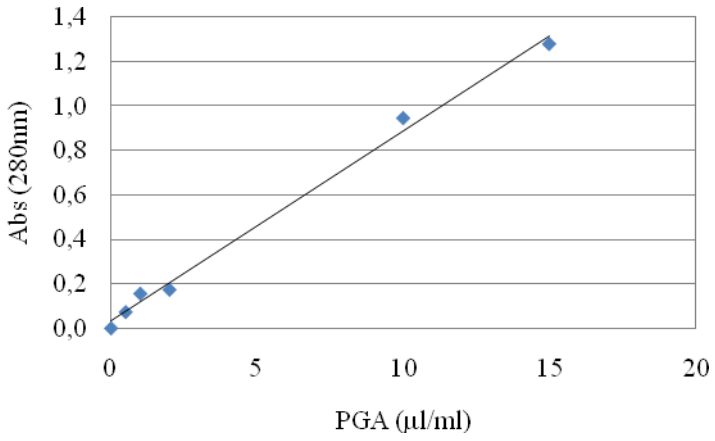

Figure 15 Calibration curve for absorbance at 280 nm of diluted PGA.

1.2. Studies of biocatalysts reutilization

Hydrolysis assays The activity of the PGA_PVA beads, obtained from 100µl of original PGA solution for a final volume of 500µl in sodium phosphate buffer solution 0.1M, pH 8 (as previously described 1.1), was measured by the pH STAT method in sodium phosphate buffer solution 20mM, pH 8 at 37ºC, during 10 minutes.

1.3. pH effect on PGA_PVA beads activity

Entrapment technique Four PGA solutions were prepared with 150µl of original PGA solution for a final volume of 500µl in sodium phosphate buffer solution 0.1M, pH 8, as previously described 1.1.

Hydrolysis assays To measure the activity, the immobilized enzyme was suspended in 20ml of sodium phosphate buffer solution 20 mM at different pHs and 37ºC, in a temperature controlled, magnetically stirred reactor. Each batch of PGA_PVA beads was suspended with different pHs; 6, 7, 8 and 9, respectively. When the suspension reached 37ºC, 5 ml of concentrated penicillin G solution, in the same buffer, at the same temperature, was added so that its final concentration became 4% (w/v) and then the activity of each batch of PGA_PVA beads was evaluated by the pH STAT method. In the end of the assays the beads are dried at 80ºC for 24h and weighed, the dry weight is used to standardize the results.

1.4. Temperature effect on PGA_PVA beads activity

Entrapment technique Five PGA_PVA beads batches were prepared with 100µl of original PGA solution for a final volume of 500µl in sodium phosphate buffer solution 0.1M, pH 8, as previously described 1.1.

Hydrolysis assays To measure the activity, the immobilized enzyme was suspended in 20ml of sodium phosphate buffer solution 20 mM, pH 8, in a temperature controlled magnetically stirred reactor. Each biocatalyst batch was thermostated at different temperatures, 25, 37, 45, 50 and 55ºC. When the suspension reached the desired temperature, 5 ml of concentrated penicillin G solution, in the same buffer, at the same temperature, was added so that its final concentration became 4% (w/v). The activity of each PGA_PVA beads batch was evaluated as described 1.1. In the end of the assays the beads were dried at 80ºC for 24h and weighed, the dry weight was used to standardize the results.

1.5. Storage stability

Entrapment technique Sixteen PGA_PVA beads batches were prepared with 100µl of original PGA solution for a final volume of 500µl in sodium phosphate buffer solution 0.1M, pH 8 as previously described 1.1.

Storage Four batches were stored at each temperature in sodium phosphate buffer solution 0.1M, pH 8 - one batch at 4ºC in a refrigerator and the remaining batches in stoves at 25, 37 and 45ºC. At 0, 24, 48 and 72 hours one of each batch was removed from the refrigerator or stoves and their activities analyzed by the pH STAT method in

sodium phosphate buffer solution 20mM, pH 8, 37ºC and a PG concentration of 4%(w/v). The weights were standardized as described 1.1.

1.6. Kinetics

Entrapment technique Four PGA_PVA beads batches were prepared with 100µl of original PGA solution for a final volume of 500µl in sodium phosphate buffer solution 0.1M, pH 8 as previously described 1.1.

Hydrolysis assays To each PGA_PVA beads batch the activity was evaluated by the pH STAT method at pH 8, 37ºC but for different PG concentrations in the reactor 1, 2, 4 and 8% (w/v). The weights were standardized as described 1.1.

2) Cefalexin Synthesis

HPLC method: 50µl of a sample was collected from the synthesis reaction media and dissolved in 200µl of sodium phosphate buffer solution 5mM, pH 7.2. 50µl of this solution was dissolved in 950µl of the same buffer containing 0.5 $g.l^{-1}$ of acetaminophen (internal standard - IS) and finally 50µl of this solution was dissolved in 450µl of HPLC dilution solution (25% acetonitrile in 2mM sodium phosphate buffer solution pH 5). 20µl of the previous solution was injected and analyzed by HPLC (Merck Hitachi L-6000 pump, Merck Hitachi D-2500 chromato-integrator, Perkin-Elmer LC90UV spectrophotometric detector, Varian ChromSpher 5C18

S250x46Column) at 214 nm in an isocratic method, adapted from De Vroom (De Vroom 2000) (Figure 16). The HPLCs eluent buffer (40% acetonitrile in 5mM sodium phosphate buffer solution 5mM, pH 3.1, containing 0.2% (w/v) SDS) ran at the flow rate of 1ml.min^{-1}. Four calibration curves, using different amounts of solutes and products, in relation to the internal standard (IS), were prepared and used to assess the concentration of 7-ADCA, PGM, phenylglycine (PheG) and CEX in the synthesis reaction media (Figure 17 to Figure 20).

Figure 16 HPLC apparatus.

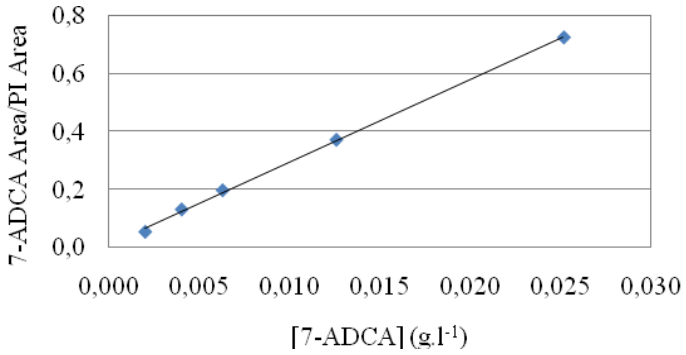

Figure 17 Calibration curve for HPLC analyses, area ratio from 7-ADCA and IS peaks versus known concentrations of 7-ADCA.

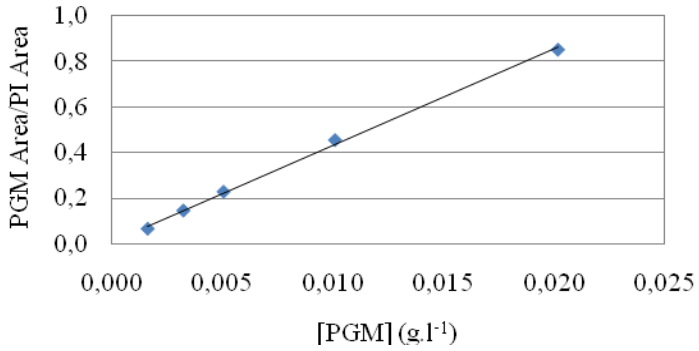

Figure 18 Calibration curve for HPLC analyses, area ratio from PGM and IS peaks versus known concentrations of PGM.

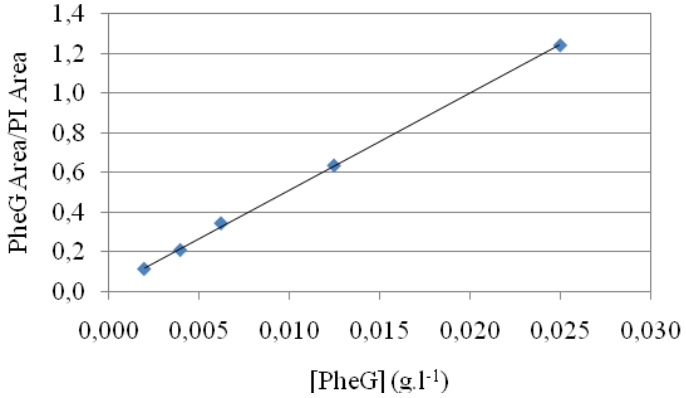

Figure 19 Calibration curve for HPLC analyses, area ratio from PheG and IS peaks versus known concentrations of PheG.

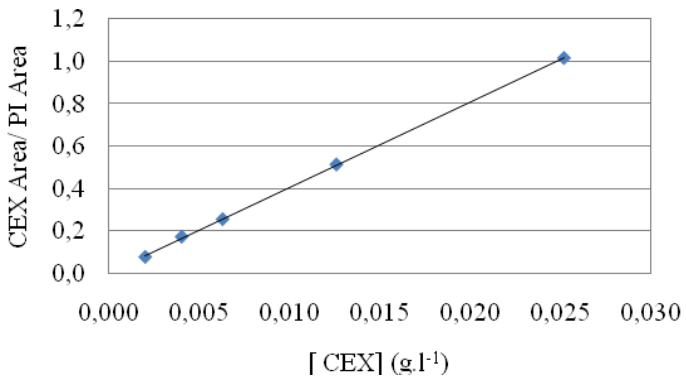

Figure 20 Calibration curve for HPLC analyses, area ratio from CEX and IS peaks versus known concentrations of CEX.

2.1. Operational stability

Entrapment technique A PGA_PVA beads batch was prepared with 100µl of original PGA solution for a final volume of 500µl in sodium phosphate buffer 0.1M, pH 8 as previously described 1.1.

Synthesis activity The immobilized enzyme beads were suspended in sodium phosphate buffer solution 5 mM, pH 7.2, in a temperature controlled, magnetically stirred reactor. The reactions were performed at 14ºC in a 25ml suspension with the initial concentrations of 30 mM 7-ADCA and 90mM PGM. The reaction ran during 8h and samples were collected at different intervals of time. The temperature of reaction was kept constant at 14ºC by cold water that flowed through the reactor jacket, using a B. Braun thermomix MM.

2.2. Kinetics

2.2.1. Maintaining a constant ratio between substrates concentrations

Entrapment technique Six PGA_PVA beads batches were prepared with 100µl of original PGA solution for a final volume of 500µl in sodium phosphate buffer solution 0.1M, pH 8 as previously described 1.1.

Synthesis activity of PGA_PVA beads was evaluated as previously described 2.1. Maintaining a constant ratio between 7-ADCA and PGM concentrations of 3 but different initial concentrations of substrates according to Table 2 the kinetic parameters of synthesis were evaluated.

Table 2 Initial concentrations of substrates in the reactor, for kinetic assays.

Assay	1	2	3	4	5	6
[7-ADCA] (mM)	5	20	50	75	100	150
[PGM] (mM)	15	60	150	225	300	450

The reaction carried out for 30 minutes and samples were collected at different intervals of time to calculate the initial velocity. For assays 1, 2 and 3, 50µl of sample were collected and dissolved in 200µl of sodium phosphate buffer solution 5mM, pH 7.2, for assays 4 and 5 the same volume of sample was dissolved in 450µl of the same buffer and for assay 6 the sample was dissolved in 700µl of the buffer, due to increasing concentrations of substrates. These diluted samples were then treated as previously described in HPLC method.

2.2.2. Maintaining one of the substrates at a constant concentration

Entrapment technique The PGA_PVA beads were prepared with 100μl original PGA solution for a final volume of 500μl in sodium phosphate buffer solution 0.1M, pH 8 as previously described 1.1.

Synthesis activity of PGA_PVA beads was evaluated as previously described 2.1. This assay study is divided in two. First, a constant initial 7-ADCA concentration of 150mM is maintained and PGM concentration varied between 5 and 120mM (5, 15, 30, 60 and 120mM) secondly; a constant initial PGM concentration of 150mM is maintained and 7-ADCA concentration varied between 5 and 120mM (5, 15, 30, 60 and 120mM). The reaction ran during 30 minutes and samples were collected at different intervals of time, to calculate the initial reaction rate. The remaining procedure was the same as previously described in HPLC method.

Discussion

1) Penicillin Hydrolysis

To calculate the penicillin hydrolysis rate, since 1 mol of phenylacetic acid is formed for each mol of hydrolyzed PG, it is necessary to maintain a constant pH in the reaction media and for this reason the pH STAT method was used, it neutralizes PAA automatically by addition of alkaline agent solution. By measuring the volume of alkaline solution spent to maintain a constant pH in the reaction media it is possible to obtain the hydrolysis rate using Equation (1).

$$\textbf{Hydrolysis rate (U)}(\mu\text{mol.min}^{-1}) = \frac{V_{NaOH}(\text{ml}) \times [NaOH](\text{M}) \times 1000}{t(\text{min})} \quad \textbf{(1)}$$

The PGA_PVA beads activity can be calculated using the hydrolysis rate since the hydrolysis of PG by mechanisms other than enzymatic catalysis is negligible in these conditions and the enzyme used is “pure”. The specific activity of immobilized or free enzyme ($\mu mol.mg_{protein}^{-1}.min^{-1}$) is obtained by the ratio of hydrolysis rate with the amount of protein in enzyme solution.

1.1. Immobilization Yield

After the entrapment of different amounts of PGA in PVA matrix, the immobilization yield was calculated by Equation (2). The encapsulation efficiency yield decreases as enzyme loading increases in the matrix (Table 3).

$$\text{Yield (\%)} = \frac{\text{Activity Immobilized enzyme } (\mu\text{mol}\cdot\text{min}^{-1})}{\text{Activity Free enzyme } (\mu\text{mol}\cdot\text{min}^{-1})} \times 100 \quad (2)$$

Table 3 Quantity of immobilized enzyme, respective immobilization yield (compared to the free enzyme) and respective activity inside the reactor.

Enzyme loading (µl) to 5 ml of LL solution	15	30	50	100	125	150
Enzyme loading ($mg_{protein}.g^{-1}_{support}$)	0.0893	0.184	0.300	0.608	0.750	0.900
Immobilization yield (%)	68 ±3.4	52 ±0.3	40 ±0.6	30 ±1.6	26 ±0.7	22 ±0.7
Activity U/ml $_{reactor}$	0.322	0.493	0.638	0.948	1.01	0.702

Enzyme loading ($mg_{protein}.g^{-1}_{support}$) was calculated by Equation (3) where *V* is the volume of PGA's original solution, *[protein]* is the concentration of that same solution and *m* the weight of the hydrated PGA_PVA beads.

$$\mathbf{EL}\left(\mathbf{mg_{protein}}\cdot\mathbf{g^{-1}}\right)=\frac{\mathbf{V}(\mu\mathbf{l})\times[\mathbf{protein}]\left(\mathbf{mg_{protein}}\cdot\mu\mathbf{l^{-1}}\right)}{\mathbf{m_{hydrated\ support}}(\mathbf{g})} \quad \textbf{(3)}$$

The decreasing immobilization yield can partly be due to the partition coefficient of PGA between water and PEG solutions (Marcos *et al.* 1998), the enzyme may be dissolving in PEG prior to PVA matrix solidification which consequently led to a loss of enzyme activity. Also, after several washing steps of the PGA_PVA beads, some amount of enzyme may have leaked into the washing buffer (sodium phosphate buffer solution 0.1 M, pH 8), this happens when the PGA_PVA beads are transferred from PEG to phosphate buffer and swelling occurs, the beads weight increases roughly 6 times, the size of the pores increase and the entrapped enzymes may diffuse into the new solvent. Furthermore there is the normal deactivation of enzyme when it is mixed in PVA solution at 40ºC and simply due to entrapment inside the beads, with an approximate size of 3mm. The size of the beads hinders substrate and product diffusion consequently increase mass-transfer resistances. All together these factors led to a decrease of immobilized enzyme activity in relation to the free enzyme. Although the yield of immobilized enzyme activity is low, it is comparable to the literature values (Fernandes *et al.* 2008) where for an enzyme load of 0.13 $mg_{protein}.g^{-1}_{support}$, the yield corresponded to 48% for immobilized inulinase activity. This means that the PVA matrix, temperature for entrapment of 40ºC and the mass-transfer resistances, due to the size of the beads, are the main influences on low yield of the PGA_PVA beads activity. Considering the yields of immobilized penicillin G acylase, from 41% to 119% in different matrices and immobilization strategies (e.g. covalent link, cross-linking, etc)

(Kallenberg *et al.* 2005) the results obtained in this work are in the range of the common values of immobilization yields obtained for this enzyme.

The values of immobilized enzyme specific activity decrease with increasing enzyme load but keep almost constant after PGA_PVA beads activity has been tested for at least three times and independent of the volume of enzyme load in LL solution (Figure 21).

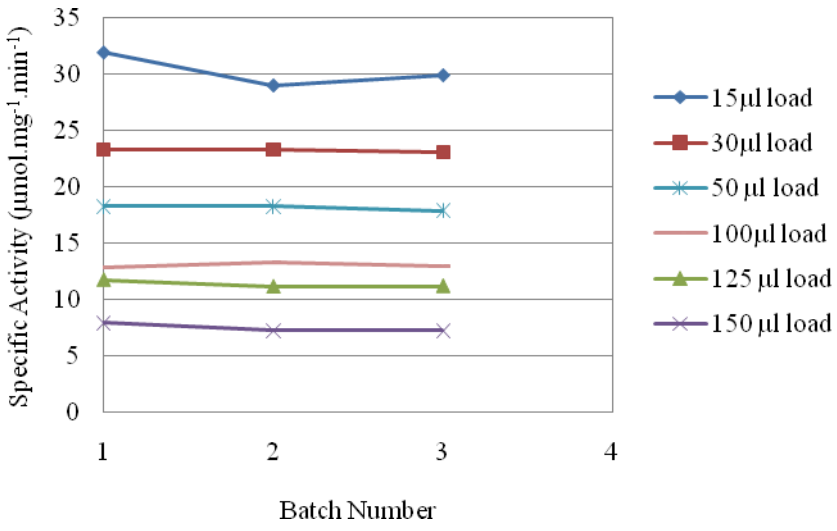

Figure 21 Specific activity of PGA_PVA beads obtained from different volume of diluted enzyme loads in 5ml of LL.

In order to achieve the best results both immobilization yield (%) and PGA_PVA beads activity inside the reactor ($U.ml^{-1}$) should be maximized. Comparing the yield with the activity inside the reactor (Figure 22), we observe that the optimum load appears to be 50 µl of PGA immobilized in 5 ml of LL solution, this value gives the best compromise between the two factors. From an industrial point of view production is usually the most

important factor; Figure 22 shows that there is an approximately linear activity increase inside the reactor until 100 µl of enzyme load, with only 10% yield loss (comparing with the previously mentioned value). For this reason, in further experiments, 0.6 $mg_{protein}.g^{-1}_{support}$ was chosen for enzyme immobilization, i.e. 100µl of diluted enzyme solution added in 5 ml of LL solution.

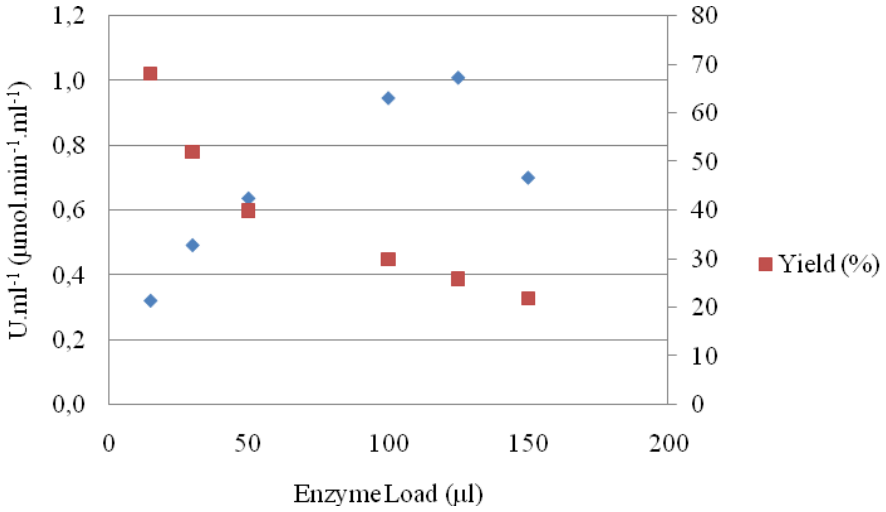

Figure 22 Comparison between yield and activity inside the reactor, with respective increasing enzyme load.

1.2. Studies of biocatalysts reutilization

To determine the capability for reutilization of PGA_PVA beads three different enzyme loads (50, 100 and 150 µl of diluted enzyme solution added in 5 ml of LL solution) were assayed in 10 consecutive PG hydrolysis reactions (Figure 23). It is clear that there is no significant loss of activity throughout 10 consecutive batches, which means that the encapsulated enzyme didn't deactivate or leak in to the supernatant.

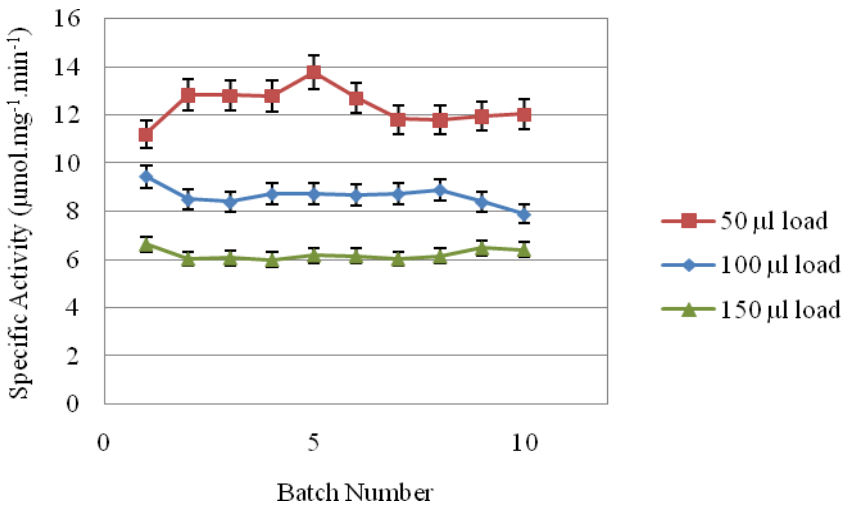

Figure 23 PGA_PVA beads activity in a sequence of 10 batches of PG hydrolysis reaction, comparison between three enzyme loads (50, 100 and 150 µl of diluted enzyme added into 5 ml of LL solution).

To confirm these results, the activity in the supernatants, after the beads were harvested, was also evaluated. The reaction of PG hydrolysis in real time was monitored by a NaOH solution consumption with an excellent linearity for the first 10 minutes of reaction, without consumption after removing the PGA_PVA beads (Figure 24).

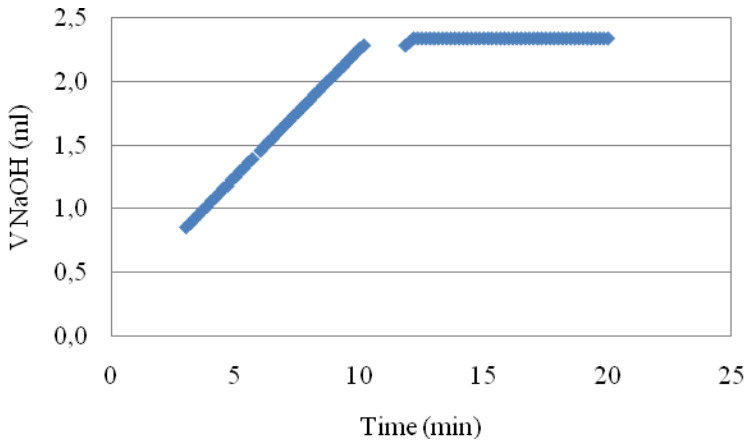

Figure 24 Volume of NaOH solution consumption versus hydrolysis reaction time, with immobilized enzyme in suspension between 1-10 min and after removal of the beads between 11-20 min, batch 1.

This proves clearly that PGA leakage from the PVA beads didn't occur. Even so, the supernatant of each hydrolysis reaction was ultra filtered until its final volume reached ~5ml and the supernatant activity evaluated by the NIPAB method. No PGA activity was detected in the supernatant by the NIPAB method, which is more sensitive than the pH STAT method.

1.3. pH effect on PGA_PVA beads activity

The microenvironment surrounding the immobilized enzyme in the interior of a carrier or support depends on specific properties of the matrix material and can affect slightly or deeply its activity and stability. The optimum pH for PG hydrolysis by the immobilized Penicillin G acylase in PVA beads is 8 (with a specific activity of 8.04 $\mu mol.mg^{-1}_{protein}.min^{-1}$) (Figure 25), which is consistent with values published in the literature (Giordano *et al.* 2006), (Plaskie *et al.* 1978). However, the plateau of optimum pH values for immobilized PGA is shorter than the observed for the free enzyme (41.0 $\mu mol.mg^{-1}_{protein}.min^{-1}$ for pH 7 and 40,7 $\mu mol.mg^{-1}_{protein}.min^{-1}$ for pH 8). The microenvironment promoted by the PVA matrix, the macro size of the beads and the diffusion limitation mentioned earlier may be the cause of the lower plateau of optimum pH for the immobilized enzyme. Additionally, PG hydrolysis produces PAA that is an acid that can interact with the PVA matrix and the enzymes microenvironment becomes more acidic than in the bulk of the reaction medium. This may occur not only because of the intrinsic limitations of diffusion inside the beads but also because the solubility of PAA in water is very low and it is likely to stay longer inside the bead than other, more hydrophilic molecules, resulting from the hydrolysis of PG, such as 6-APA. The

decrease of the immobilized activity at pH 9, may be due to some enzyme deactivation in the interior of the matrix, at higher pH values. Interaction with the matrix may occur and alter slightly the enzymes conformation, but enough to decrease its activity.

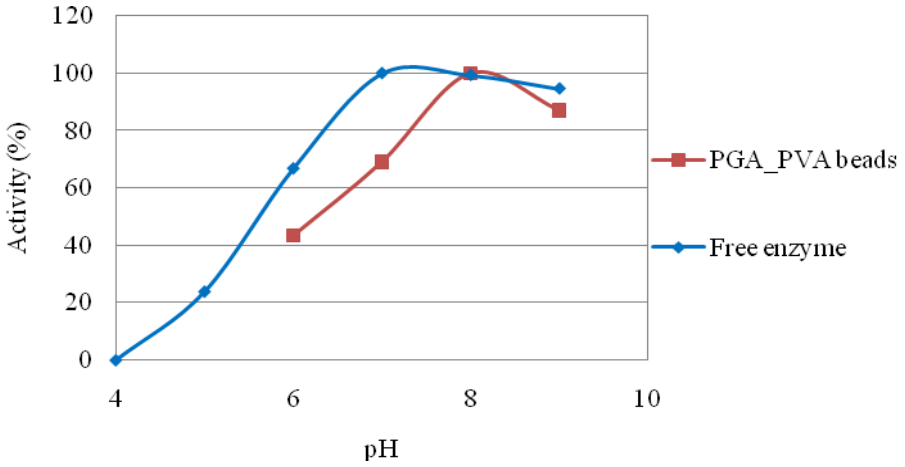

Figure 25 Relative activity at different values of pH for the free enzyme (max - 41.0 µmol.mg$^{-1}_{protein}$.min^{-1}) and the PGA_PVA beads (max - 8.04 µmol.mg$^{-1}_{protein}$.min^{-1}).

1.4. Temperature effect on PGA_PVA beads activity

There is a peak of activity at 45ºC both in free and immobilized enzyme (Figure 26) which corresponds to 51.6 µmol.mg$^{-1}_{protein}$.min^{-1} and 23.9 µmol.mg$^{-1}_{protein}$.min^{-1}, respectively. For temperatures bellow 45ºC the curves have a similar behavior but for temperature values above 45ºC the immobilized enzyme destabilizes sharply and loses its activity faster than the free enzyme.

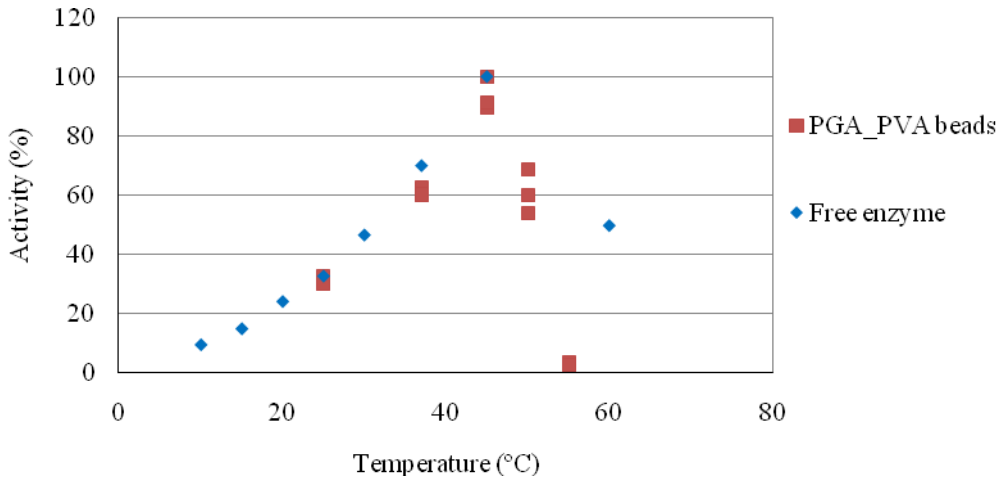

Figure 26 Relative activity at different temperatures for the free enzyme (51.6 µmol.mg$^{-1}_{protein}$.min^{-1}) and the PGA_PVA beads (23.9 µmol.mg$^{-1}_{protein}$.min^{-1}).

The activation energy (E_a) was calculated from the slope of the graph plotting the natural logarithm of the initial velocity ($\ln V_0$) versus the inverse of the absolute temperature (1/T) according to the Arrhenius equation (Figure 27).

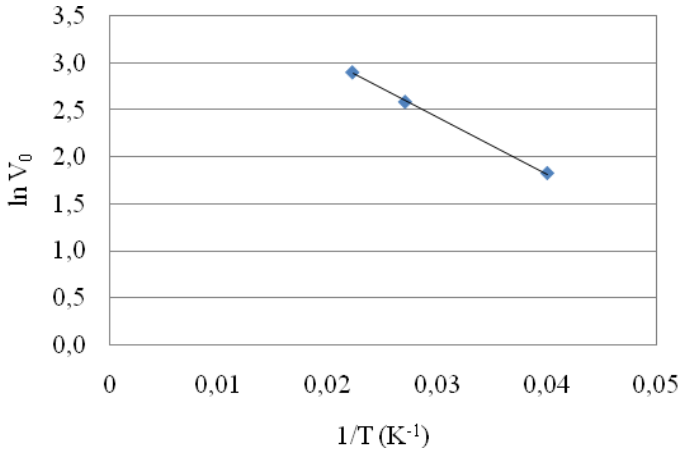

Figure 27 Arrhenius linearization of the PGA_PVA beads activity values at different temperatures, in order to calculate the activation energy (Ea).

The obtained value of Ea was 0.5 KJ/mol for the immobilized PGA; this means that the beads activity is not very dependent on temperature changes. The E_a for the free enzyme 44.8 $KJmol^{-1}$ shows a much higher dependence on temperature. This difference in values suggest that the enzyme inside of the PVA matrix does not have enough space to move freely, limiting the conformational changes of the enzyme. This affects not only the enzyme stability but it can also explain why the activity of the PGA_PVA beads drops sharply for temperatures higher than 45ºC. The PVA matrix is stable at 55ºC at acid pH values (Fernandes *et al.* 2008) but at basic pH it starts dissolving at about 45ºC. So, for temperatures higher than 45ºC at pH 8, the PVA matrix is no longer stable. As previously mentioned (1.3) the matrix dissolution may be interacting with the enzyme and causing a destabilizing effect which induces the steep loss of activity. In these experiments the activity was measured three times for 10 minutes and the beads where kept during 90 minutes at each temperature, so not only the activity was analyzed but also its stability, for short periods of time. After the first batch at 45 and 50ºC a decrease in activity is observed (10 and 21%, respectively) and at 55ºC there was practically no activity.

In spite of the optimum temperature being 45ºC the preferential temperature for hydrolysis studies is 37ºC since there is no influence of enzyme denaturation during short periods of time.

1.5. Storage stability

The immobilized enzyme was stored at different temperatures and its stability analyzed using the linear inverted, Equation (4) and exponential Equation(6), models (Cardoso *et al.* 1978). At 4ºC the activity remains stable throughout the 72 hours of the assay, at 25ºC there is a tendency of the immobilized enzyme to lose activity and at 37ºC this is very noticeable (Figure 28). At 45ºC the activity loss is quite harsh and at the end of the first day under storage at this temperature there is no more enzymatic activity (Figure 26).

$$\mathbf{E(t) = \frac{E_0}{1 + k_d t}} \quad \textbf{(4)}$$

$$t_{\frac{1}{2}} = \frac{1}{k_d} \quad \textbf{(5)}$$

$$\mathbf{E(t) = E_0 e^{(-k_d t)}} \quad \textbf{(6)}$$

$$t_{\frac{1}{2}} = \frac{\ln 2}{k_d} \quad \textbf{(7)}$$

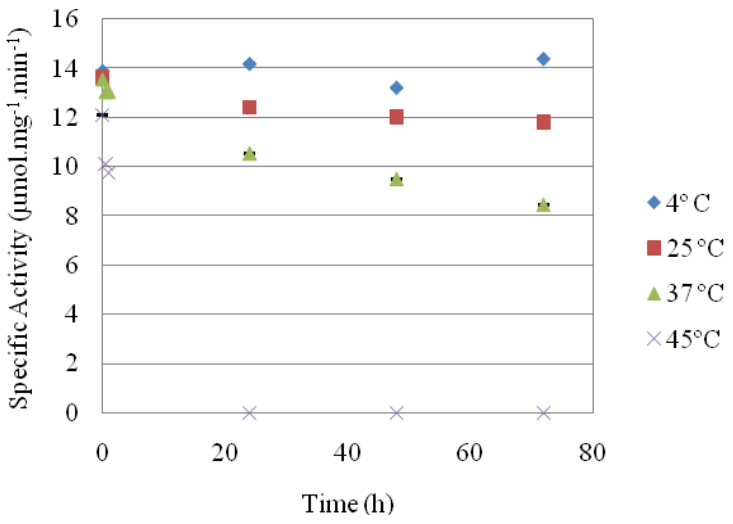

Figure 28 Storage stability of Immobilized PGA at different temperatures.

Both linear inverted and exponential models were analyzed for thermal stability of immobilized enzyme and to simulate the obtained values. It was not possible to rule out one of them, they both fit the data with similar correlation values, the linear inverted model is slightly better than the exponential model but the difference isn't significant (Table 4). For 25ºC the half life ($t_{1/2}$) of the immobilized enzyme, calculated for the linear inverted and exponential model by Equation (5) and Equation (7) respectively, is over 20 days, 5 days for 37ºC but only about 1 hour when the enzyme is at 55ºC.

Applying the Arrhenius equation, Equation (8) for immobilized enzyme deactivation and plotting ln(k_d) versus the inverse of the absolute temperature (1/T) (Figure 29 and

Figure **30**) the thermal deactivation energy (E_d) was calculated (Table 4). E_d for the immobilized penicillin G acylase is ~ 175 KJ.mol^{-1} for both models; this means that the

deactivation of the PGA_PVA beads is highly dependent on temperature, contrary to its activation energy, according to the Arrhenius Equation.

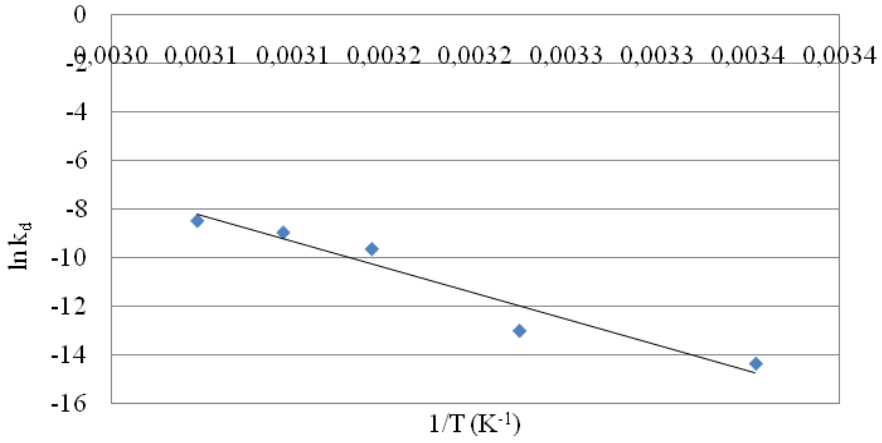

Figure 29 Arrhenius linearization of the PGA_PVA beads activity values at different temperatures using k_d values calculated by the linear inverted model.

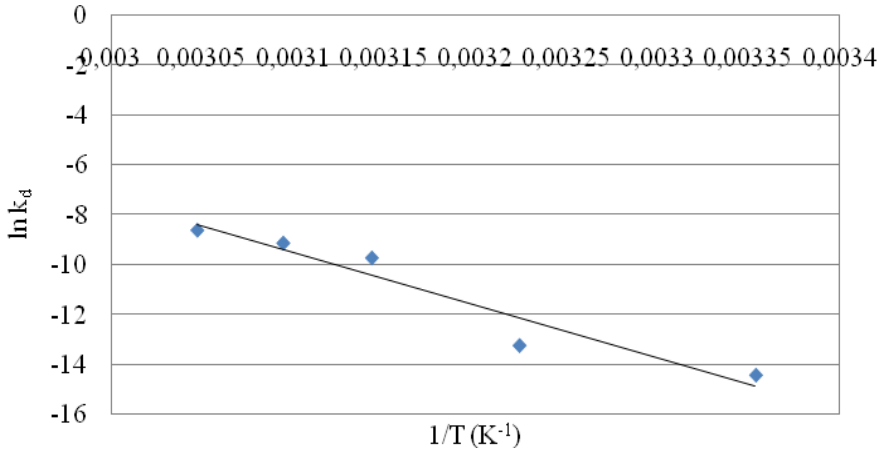

Figure 30 Arrhenius linearization of the PGA_PVA beads activity values at different temperatures using k_d values calculated by the exponential model.

The thermal deactivation energy for the PGA_PVA beads is lower than the free enzymes which is 236 KJ.mol^{-1} (Pan *et al.* 2004), this means that it is easier to denature PGA, by temperature increase, when immobilized in PVA/LL than the free enzyme. As observed in (1.3), at alkaline pH values, the PVA matrix isn't very stable and this fact when analyzed with the temperature increase is disturbing the enzymes microenvironment, destabilizing it.

$$\ln(k) = \ln(A) - \frac{E_d}{RT} \quad (8)$$

Table 4 Values of deactivation constants, half life and deactivation energy for PGA_PVA beads, calculated by the linear inverted and exponential models.

Temperature		Linear inverted model			Exponential model		
K	**ºC**	$k_d(s^{-1})$	$t_{1/2}(h)$	η^2	$k_d(s^{-1})$	$t_{1/2}(h)$	η^2
298	**25.0**	5.78 x10^{-07}	481	0.881	5.36 x10^{-07}	359	0.870
310	**37.0**	2.25 x10^{-06}	123	0.976	1.76 x10^{-06}	109	0.952
318	**45.0**	6.65 x10^{-05}	4.18	0.888	5.96 x10^{-05}	3.23	0.878
323	**50.0**	1.32 x10^{-04}	2.10	0.990	1.08 x10^{-04}	1.78	0.977
328	**55.0**	2.14 x10^{-04}	1.30	1.00	1.81 x10^{-04}	1.06	1.00
E_d (KJ/mol)		176			174		

1.6. Kinetics

In order to obtain the kinetics of the immobilized enzyme on the hydrolysis of PG, used as a natural substrate (Figure 32), several experiments were performed to determine V_{max} and the apparent K_M using the linearization of Lineweaver-Burk (Figure 32) and Eadie-Hofstee (Figure 33). The initial velocity of the reaction was obtained during the first 10 minutes of reaction, with very little products formation (6-APA and PAA). The PGA_PVA beads show a typical Michaelis-Menten kinetic behavior on the hydrolysis of PG and until 80g.l^{-1} of PG, inhibition by the substrate or product was not observed (Figure 31), in spite of PAA being a competitive inhibitor for PGA, and being able to induce conformational changes in the enzyme (Janssen *et al.* 2001).

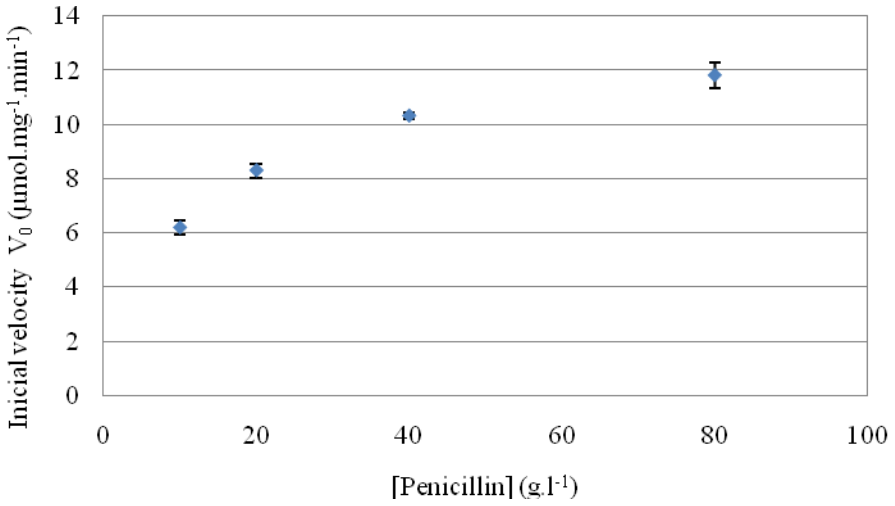

Figure 31 Variation of initial velocity on PG hydrolysis by PGA_PVA beads with increasing substrate (PG) concentration.

The obtained K_M and V_{max} values calculated according to the Lineweaver-Burk and Eadie-Hofstee linearization methods (Table 5) for the immobilized PGA are ~11.8 g.l^{-1} (27 mM) and ~13.5 µmol.mg$^{-1}_{protein}$.min^{-1}, respectively. The K_M values indicate the

enzymes affinity towards the substrate; the lower these values, the higher the affinity, which means that the maximum catalysis velocity is achieved at lower substrate concentrations. In the literature the K_M value for the free enzyme is 5.99 mM (37ºC, pH7.9) and 29.8mM when the enzyme is immobilized in Sepabeads EC-EP carrier (Žuža *et al.* 2007), the calculated value for the PGA_PVA beads is about 5 times higher than the free enzyme, this is due to the diffusion limitations inside the bead and mass-transfer resistance of substrates from bulk to bead surface, or vice–versa for products generated. The microenvironment surrounding the enzyme is different to the bulk solution, the substrate concentration, actually felt by the enzyme, is lower than its real concentration in the reaction medium due to internal and external mass-transfer resistance of substrates and products, caused by the enzyme immobilization in PVA matrix. So, when enzymes are immobilized, the K_M value and its real meaning are substituted by the apparent $K_{M.}$

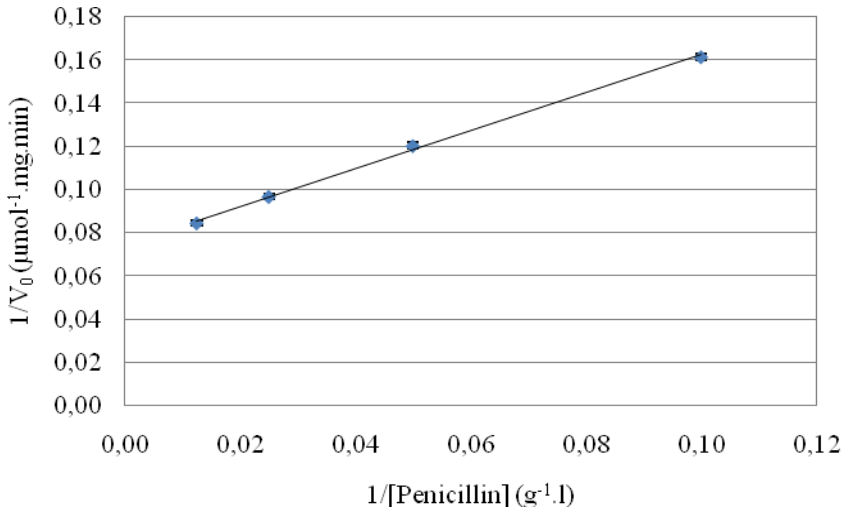

Figure 32 Lineweaver-Burk linearization, obtained from the data of Figure 31.

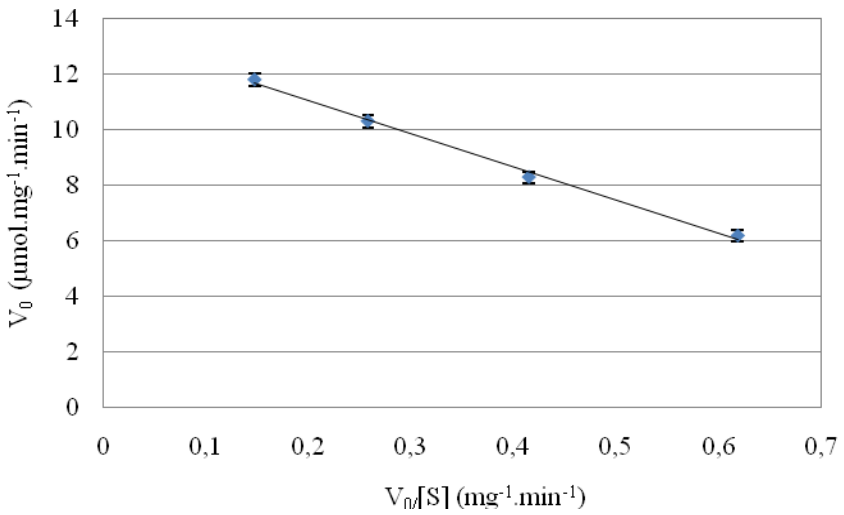

Figure 33 Eadie-Hofstee linearization, obtained from the data of Figure 31.

Table 5 Values of V_{max} and apparent K_M obtained from Lineweaver-Burk and Eadie-Hofstee linearization of Figure 31.

Linearization technique	**Lineweaver-Burk ($1/V_0$ vs $1/[S]$)**	**Eadie-Hofstee (V_0 vs $V_0/[S]$)**
Apparent K_M ($g.l^{-1}$)	11.8 ± 0.33	11.9 ± 0.54
V_{max} ($\mu mol.mg^{-1}_{protein}.min^{-1}$)	13.5 ± 0.25	13.4 ± 0.21

2) Cefalexin Synthesis

Penicillin G acylase is widely used to hydrolyze penicillin G, from this deacylation results the β-lactam nucleus (6-APA) that is used on chemical synthesis of semi-synthetic β-lactam antibiotics (e.g. ampicillin and amoxicillin). PGA can also synthesize

these antibiotics when in organic solvents or reaction media with low water activity and in the presence of the adequate substrates, 6-APA and activated phenylglycine derivatives in lab scale. This enzyme is already used in the industrial synthesis of other semi-synthetic cephalosporin antibiotics, such as cefalexin, from 7-ADCA and activated phenylglycine derivatives (PGM or PGamide) (Wegman *et al.* 2001). These manufacture enzymatic processes allow substitution of deacylation but also the condensation chemical processes. New enzyme immobilization techniques need yet to be developed so that it becomes economically viable and successfully substitute the chemical synthesis by biochemical processes. Additionally, the yield of kinetically controlled reaction has to be maximized by the improvement of the synthesis vs hydrolysis ratio by a smart choice of matrix for immobilization and optimization of reaction conditions.

2.1. Operational stability

To verify the stability of the immobilized enzyme during the reaction of cefalexin synthesis the operational stability was measured for over 50 hours (Figure 34). The reaction carried out at 14ºC in 10 consecutive batches and the initial velocity was measured during the first 30 min of cefalexin synthesis. The initial velocity of each batch does not significantly change and the activity of PGA_PVA beads is stable around a value of 1.8 $\mu mol.mg^{-1}_{protein}.min^{-1}$, when operating for at least 50 hours.

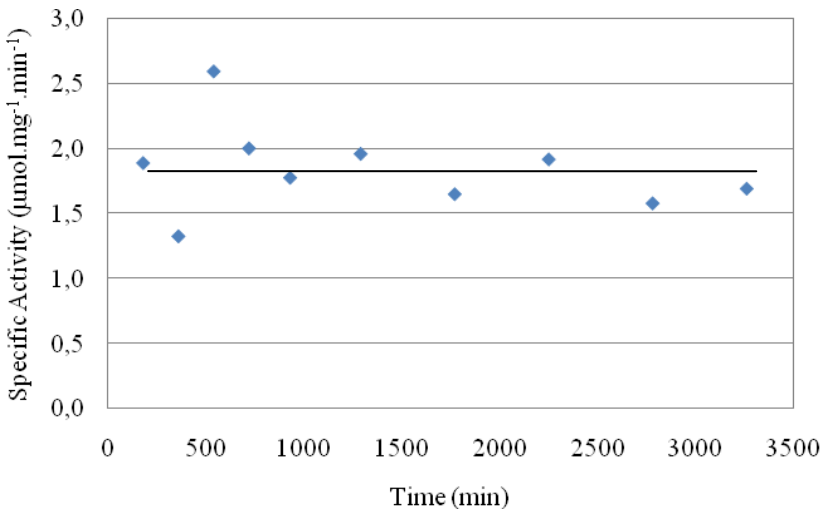

Figure 34 Operational stability of PGA_PVA beads during cefalexin synthesis through 10 consecutive batches. The initial velocity was measured for each batch during 30 min.

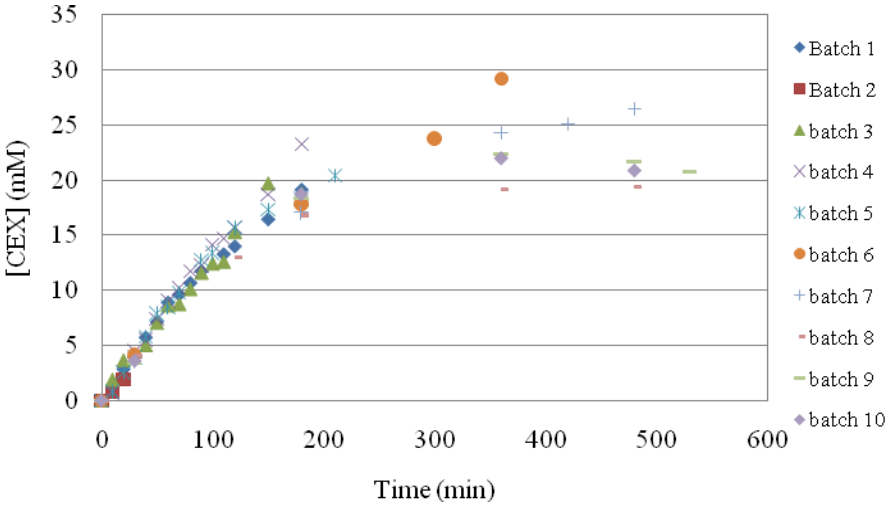

Figure 35 Cefalexin concentration along the reaction, synthesis of cefalexin by PGA_PVA beads, 10 batches.

By comparison, for all 10 consecutive batches it is possible to see an excellent concordance of the cefalexin concentration for the first 3 hours of reaction (Figure 35),

afterwards the data start showing some slight dispersing, achieving a maximum concentration of 23 mM after 8 hours of reaction, which corresponds to a conversion yield of 76% in relation to the limiting substrate (7-ADCA) (Figure 36).

The reaction yield was calculated using the ratio between the concentration of product (cefalexin) and the concentration of the limiting substrate (7-ADCA), Equation (9).

$$\mathbf{Yield(\%)} = \frac{\mathbf{[CEX]}}{\mathbf{[7-ADCA]}} \times \mathbf{100} \quad \textbf{(9)}$$

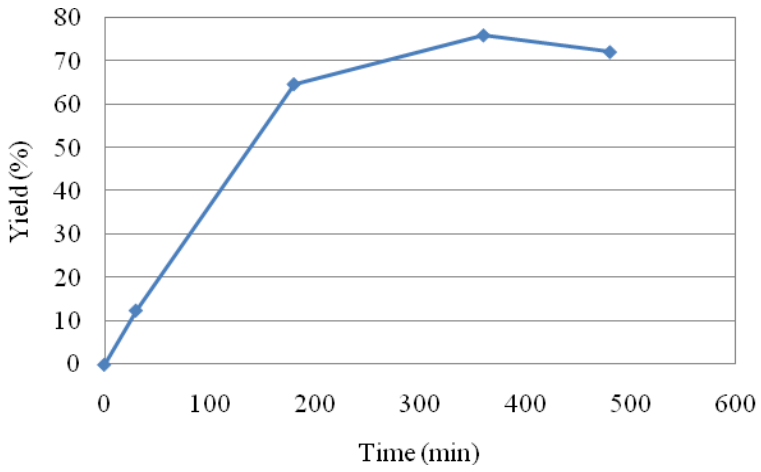

Figure 36 Reaction yield of cefalexin synthesis along the reaction, example obtained from batch nº 10.

This maximum value of cefalexin concentration is the result of products accumulating in the beads and the substrate becoming rare, the synthesis velocity diminishes and the hydrolysis velocity increases and attains equal values at equilibrium. In contrast with some other examples of PGA used in synthesis of cefalexin this immobilization method

does not show significant hydrolysis and degradation of cefalexin accumulated in the reaction media (Figure 35 and Figure 36).

The concentration of PGM is, initially about 3 times higher than 7-ADCA's, this induces the reaction equilibrium to shift and benefit product formation but PGM can also be a substrate for PGA, resulting in its hydrolysis in PheG and methanol, Equation(10) so, there is not only an increase in the synthesis of cefalexin, but also an increase in hydrolysis of PGM (Figure 37). When the limiting substrate concentration (7-ADCA) starts decreasing and achieves the reaction equilibrium practically no more CEX is synthesized but the hydrolysis of PGM continues, as expected, and the PheG concentration increases in the reaction media (Figure 37).

$$\mathbf{PGM} \overset{\mathbf{PGA}}{\longleftrightarrow} \mathbf{PheG + MeOH} \qquad \textbf{(10)}$$

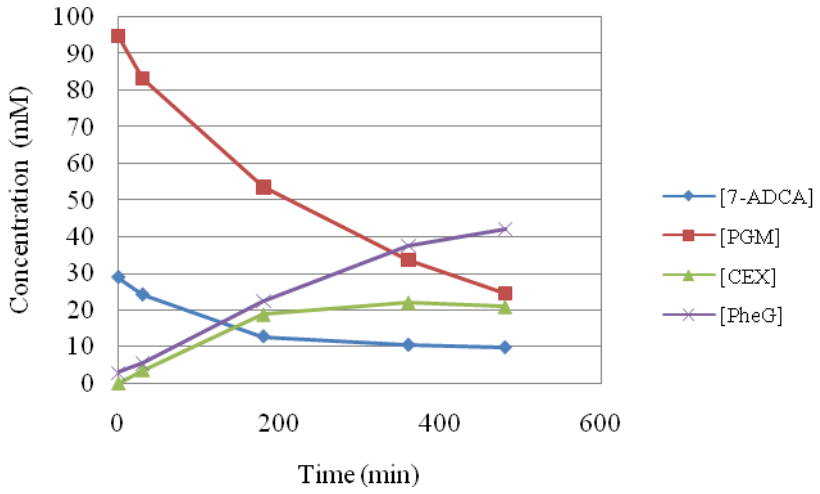

Figure 37 Substrates and products concentration along the reaction, example obtained from batch nº 10.

The most common way to classify whether a catalyst is adequate for synthesis is calculating the ratio between synthesis of CEX over hydrolysis of PGM rates for a given reaction time Equation (11).

$$S/H = \frac{[CEX]}{[PheG]} \quad \textbf{(11)}$$

S/H was calculated using the initial velocity of formation of these two products, cefalexin and phenylglycine, in the first 30 min of reaction. For the fee enzyme, at 14ºC and pH 7.2 an S/H ratio of 4.2 (data not shown) was obtained and for the PGA_PVA beads the value was ~ 2.5 (Figure 38) which is similar to values presented in the literature for immobilized enzymes. The production of CEX from D-phenylglycin nitrile and 7-ADCA by a two-enzyme cascade, nitrile hydratase and PGA, was achieved with a 60% yield and an S/H ratio of 2.7 (Wegman *et al.* 2001). Schroën *et al.* compared the yield and S/H ratios of free PGA and the commercial immobilized PGA enzyme, Assemblase 3750® and found that at pH 7.5, 100 mM (equimolar substrate) and 18ºC, the yields are 82% and 78% and the S/H ratios are 2.1 and 1.1, respectively. The yield is not very affected by enzyme immobilization but, on the other hand the S/H ratio doubles when the enzyme is free in solution. S/H ratio is known to be dependent on temperature, pH, substrate concentration, cosolvents, etc (Schroen *et al.* 2002), (Illanes *et al.* 2007), (Illanes *et al.* 2005), (Illanes *et al.* 2004). An already used technique to

increase this ratio is to precipitate and remove the product, decrease its concentration and shift the reaction equilibrium towards the synthesis of the products, by adding a precipitant to the two-enzyme cascade, mentioned above, the S/H ratio was increased to 7.7 with a 79% yield (Wegman *et al.* 2001).

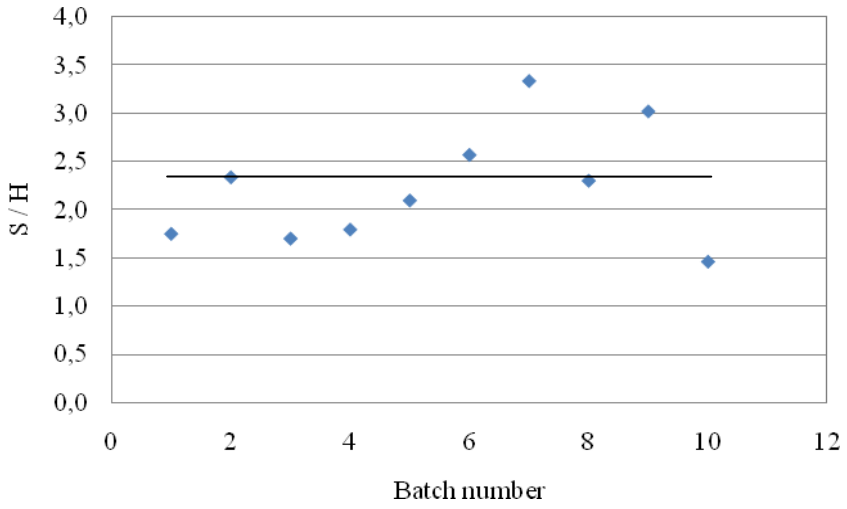

Figure 38 S/H ratio, the velocities of synthesis and hydrolysis ratio in the first 30 minutes of reaction, for 10 consecutive batches of the same PGA_PVA beads, on the synthesis of cefalexin.

2.2. Kinetics

2.2.1. Maintaining a constant ratio between substrates concentrations

In order to understand the relation between PGA activity and the substrates concentrations involved in the synthesis of cefalexin the variation of 7-ADCA and PGM concentrations was studied. It is current that, to produce cefalexin, PGM is used in a concentration corresponding to, at least, 3 folds 7-ADCAs concentration (Illanes *et al.*

2007). This increases the reaction rate and the kinetically controlled reaction is favored, for that reason the same ratio is maintained in this study. PGA inhibition occurs for [7-ADCA] > 100mM and [PGM] > 300mM, for the free enzyme (Figure 39).

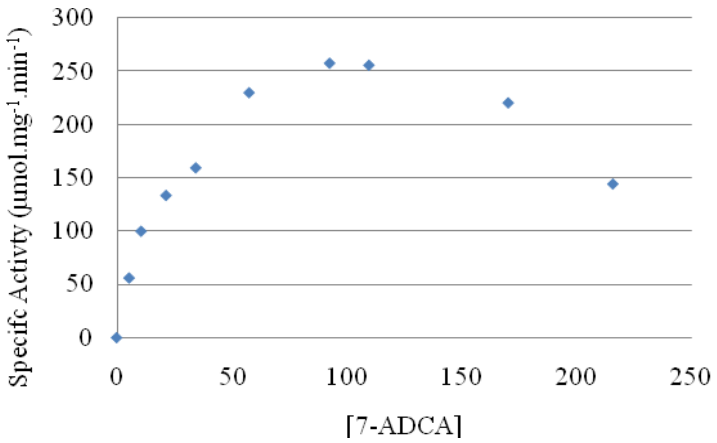

Figure 39 Variation of the free enzyme initial specific activity with increasing substrate concentrations of 7-ADCA and PGM, maintaining the ratio of 3X folds the [PGM] for each [7-ADCA].

Since the substrates concentrations ratio was maintained constant and equal to 3 it wasn't possible to know which is causing inhibition. This inhibition effect is not observed for the immobilized enzyme in PGA_PVA beads, in the same concentrations range (Figure 40). This may be due to the previously mentioned diffusion problems inside the beads, diffusion is slower in relation to bulk solution and the enzymes microenvironment inside the PVA beads is different, which affects also its inhibition patterns, this means that the substrates concentration next to the enzyme is lower and so inhibition is prevented through a longer concentration interval.

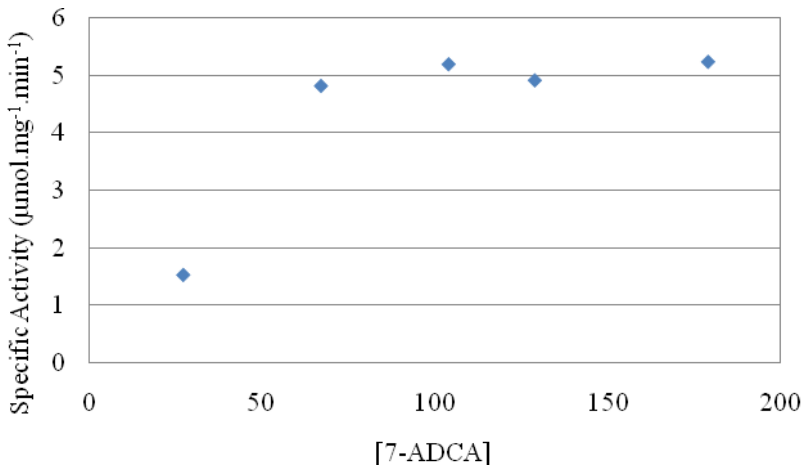

Figure 40 Variation of the PGA_PVA beads initial specific activity on synthesis of CEX with increasing substrate concentration of 7-ADCA, maintaining the ratio of 3X fold the [PGM] for each [7-ADCA].

2.2.2. Maintaining one of the substrates at a constant concentration

To clarify this inhibition effect for the two substrates used in the synthesis of CEX it is necessary to analyze PGA activity by varying substrate concentration. One substrate concentration is fixed while the other varies and this is done twice, inversing the substrate that is fixed. With these experiments it is possible to see individually, for each substrate, its inhibiting action on the PGA_PVA beads. Analyzing Figure 41 we observe that PGA activity is not very sensitive to PGM and the reaction of synthesis only occurs at [PGM] > 60 mM for a 7-ADCA concentration of 150 mM but this concentration was selected before knowing which substrate was inhibiting the enzyme and how strong was the inhibition effect on enzyme activity. When PGM was fixed at 150 mM, at 30 mM of

[7-ADCA] there is a maximum of activity and at 60mM the activity decreases, indicating a strong inhibition on PGA activity by this substrate (Figure 42).

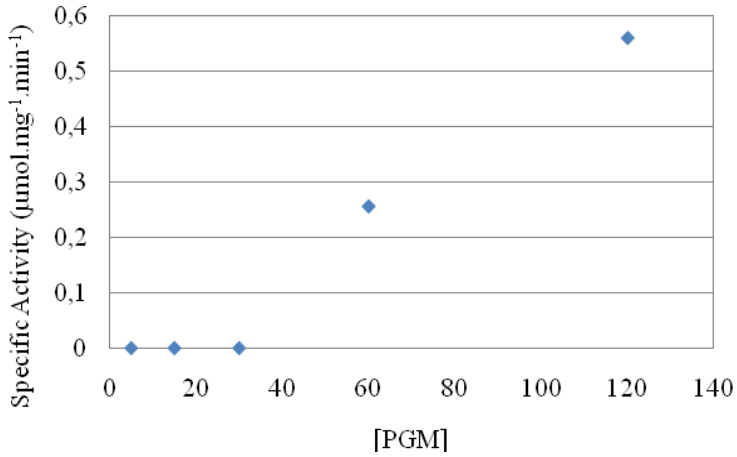

Figure 41 Variation of the PGA_PVA beads initial specific activity on synthesis of CEX related to concentration of PGM, maintaining 7-ADCA concentration constant at 150mM.

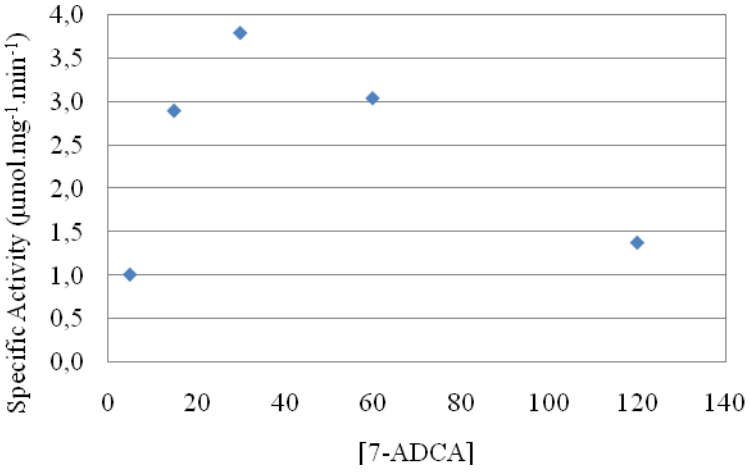

Figure 42 Variation of the PGA_PVA beads initial specific activity on synthesis of CEX related to concentration of 7-ADCA, maintaining PGM concentration constant at 150mM.

There is also the fact that maintaining the PGM concentration and increasing 7-ADCAs concentration the ratio between them is changing and the activity also diminishes. The immobilized activity is not only lowered by inhibition but also by ratio changes between the substrates.

Concluding remarks and Future work

This work described the immobilization of Penicillin G acylase in a PVA/LL based matrix. The characteristics of this new biocatalyst, the PGA_PVA beads, were studied for the hydrolysis of Penicillin G and the synthesis of Cefalexin from phenylglycine methyl ester and 7-aminodesacetoxicefalosporanic acid. Although throughout this work the calculated values for the free enzyme are usually better than the ones for the immobilized enzyme, mostly due to diffusion limitations inside the beads, these data are only used for comparison. Industrially the application of free enzymes makes processes unviable due to enzyme recovery difficulties. For the hydrolysis the best results were obtained at 37ºC, pH 8 and low enzyme loading. The PGA_PVA beads show a high stability when operating, maintaining its initial activity for at least 10 batches, for the hydrolysis and 50 hours of operation for the synthesis reaction. There are already many different immobilization techniques that are suitable for industrial application of the enzymatic hydrolysis of PG into 6-APA but few techniques are applicable in the synthesis of CEX. The enzymatic pathway to produce CEX is still in need of improvement and so the chemical synthesis is not yet completely substituted, in order for this to happen the final concentration of product in the reactor and the S/H ratio must be improved. An S/H ratio of ~2.5 was achieved with a 77% yield for the PGA_PVA beads, pH 7.2 in water media at 14ºC. These results are similar to others obtained by biocatalysts already used in industry and can be further improved by optimization of enzyme loading and product precipitation during the synthesis reaction. The mild reaction conditions, stability, simple immobilization method, cheap carrier and

manageability make PGA_PVA beads easily applicable in industry and a new option for the production of cefalexin.

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Glossary

6-APA - 6-aminopenicillanic acid

7-ADCA - 7-aminodesacetoxycephalosporanic acid

CEX - cefalexin

E_a – activation energy

E_d – deactivation energy

IS - Internal standard, acetaminophen

LL - Lentikat® Liquid

NABA - 3-amino-6-nitrobenzoic acid

NIPAB - 6-nitro-3-phenylacetamide benzoic acid

PAA - phenyl acetic acid

PEG - polyethyleneglycol 600

PG - phenylglycine

PGA - Penicillin G acylase

PGA_PVA beads - PGA biocatalyst, The enzyme is immobilized in a PVA-based matrix

PGM - (R)-(-)-2-phenylglycine methylester hydrochloride

Specific activity - ($\mu mol.mg^{-1}_{protein}min^{-1}$) was calculated by the ratio between the velocity of the reaction ($\mu mol.min^{-1}$) and the amount of enzyme (mg)

U - One unit of activity (U) is defined as the amount of enzyme required to produce 1μmol of product per minute.